EXPERIMENTAL CELL RESEARCH

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MICROSPECTROPHOTOMETRY OF CELL NUCLEI STAINED WITH FEULGEN REACTION

III. THE DNA CONTENT OF INDIVIDUAL NUCLEI OF RAT TISSUES IN THE POSTNATAL GROWTH

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The concept of the constancy of DNA content per set of chromosomes in a given species, first reported by Boivin, Vendrely and Vendrely [2] and Mirsky and Ris [24] on the basis of chemical determination of the average DNA content of nuclei, was amply confirmed at the level of individual nuclei by different workers using the technique of microspectrophotometry [53, 54, 22, 20, 11, 55]. In some instances, however, apparent discrepancy from this rule has emerged from microspectrophotometric studies on different materials [46, 19, 17, 25, 34, 35, 36, 37]. Some of them were criticized or disproved by other groups of workers [23, 54, 1]. Most biologists, however, seem to be inclined to accept the original concept of the DNA constancy rule with some qualifications which may be restated somewhat like this: *In most cases* the *average* DNA content of the nuclei in a given species is *approximately proportional to the ploidy* and is *not affected by the metabolic conditions* of the organism; it is admitted that there may well be a certain degree of fluctuation around the modal value presumably due to aneuploidy [18, 19, 20, 21, 32, 55, 58].

Since simplified apparatus of microspectrophotometry working in visible range was introduced by Pollister *et al.* [41, 42] and Lison [15], a great number of articles have been published concerning the determination of DNA content of cell nuclei of different materials using this technique. Naora [26, 27, 28, 32] pointed out a possibility of involvement of the Schwarzschild–Villiger effect (S-V effect) in the equipments hitherto employed and devised an improved apparatus which was constructed so as to eliminate this effect. Theoretically, the S-V effect in the photometric system is expected to yield a serious error in the determination of transmittance, when objects of higher optical densities are being measured. The magnitude of the error introduced

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thereby was shown with several examples. Naora's idea was challenged by Ornstein and Pollister [33], but they were again criticized by Lison [16] and Naora [28]. Thomson and Frazer [55] have obtained the results involving the S-V effect in their experiment, but suggested that with their system the S-V effect did not cause any significant error in measurement of Feulgen dye per nucleus. It is evident that the validity of the microspectrophotometric measurement reported so far, without precautions to eliminate the S-V effect, is problematic though it may not be entirely untenable.

The objective of the present study is to compare the amount of the Feulgen dye developed in individual nuclei as a relative measure of DNA per nucleus (1) among cells of a given type at different stages of postnatal growth of the rat, using several kinds of somatic cells; and (2) among different types of cells in one and the same individual at varying growth stages. Integration of the results obtained strongly suggests that the rule of DNA constancy per set of chromosomes holds for different tissue cells of individual animals, but the DNA content per nucleus of a given ploidy class is subject to variation which may possibly be related to the course of the postnatal growth.

MATERIAL AND METHOD

Male albino rats of unspecified strains, mostly taken from Sibatani's laboratory colony, served as material. They had been inbred for generations by brother–sister mating.

The optimum conditions of tissue preparation and Feulgen reaction for microspectrophotometry were established by a series of pilot experiments [31]. These experimental conditions were used in the present study. Rats of different postnatal growth stages, ranging in body weight from 11 up to 400 g, were killed by decapitation and livers and several other organs were fixed, if not otherwise stated, in 50 per cent formalin for 3 hours. In some experiments, alcohol–formalin (9:1; overnight) was employed as the fixative for comparison. Specimens to be compared were fixed and embedded in paraffin at the same time, and the representative sections of appropriate thicknesses (6–16 μ) were adhered on one large coverslip, subjected to Feulgen reaction as described below, dehydrated and mounted in balsam with another but thin time smaller coverslip.

Sections were hydrolysed in N HCl for 13 minutes at 60°C [31], and the Feulgen colour was developed using three kinds of the reaction mixtures [52].

F: Conventional Schiff reagent, prepared according to the original procedure of Feulgen and Rossenback [8], with slight modification as indicated by Sibatani and Fukuda [50]; reaction time 60 or 100 minutes.

BM: 8.5 ml Sörensen's 0.1 M glycine buffer of pH 2.28, 1 ml 15 per cent aqueous sodium metabisulfite, and 0.5 ml Schiff reagent; reaction time 3 hrs.

B: 9.5 ml of the same glycine buffer as used in BM and 0.5 ml Schiff reagent; reaction time 4 hrs.

BM and B were prepared freshly from stock solutions immediately before use. The effect of these different reaction mixtures on the optical properties of the Feulgen dye produced *in situ* as well as *in vitro* was submitted in Sibatani and Naora's papers [47, 48, 52]. For removal of excess Schiff reagent, two kinds of the SO_2-containing solution were employed:

A: Remarkably acidic mixture as used conventionally since Feulgen and Rossenbeck [8].
B: Buffered mixture as recommended elsewhere [51].

No attempts were made in this study to obtain a constant colour intensity of the Feulgen reaction *in situ*. On the contrary, the intensity of the Feulgen reaction was varied on purpose by using different reaction mixtures and rinsing solutions on the one hand, and Schiff reagents aged during different lengths of time without any appreciable recolorisation on the other hand. This was to check whether there is any trend of the relative value for colour yield per nucleus to be affected by the actual reaction intensity, which might have been expected if some interference, due to nuclear proteins or other factors, to the histochemical Feulgen reaction had been operative in Sibatani reaction system [48, 49, 52]. In many instances one and the same material received different treatments. In presenting the results, determinations performed with individual sets of different tissues shall be designated numerically as Expt. 1, Expt. 2, etc., and different treatments given to the same set of tissues shall be distinguished alphabetically as Expt. 1 A, Expt. 1 B, etc. Thus, the actual figures for the quantity of Feulgen dye per nucleus can be directly compared within one and the same experiment alone, and only the relative values should be compared among separate experiments.

Microspectrophotometric determinations of the quantity of Feulgen dye per nucleus (expected to be proportional to DNA per nucleus) were performed with the apparatus of the Koana–Naora type designed to eliminate the S-V effect [26, 27, 28]. The details of the measuring procedure will be published [29]. Relative amount of absorbing substance per spherical nucleus is given, with this method of determination, by r^2E, where r is the radius in μ and E the extinction of the nucleus as measured according to Naora [29].

RESULTS

Two types of experiments were performed. First, the change in quantity per nucleus of the Feulgen dye developed under identical conditions was followed in the course of postnatal growth of rats with several kinds of tissue cells. Studies were conducted on the cells of liver parenchyma, of adrenal medulla, of uriniferous tubules of kidney cortex, and of stratum granulosum of cerebellum. Measurements on liver cells and other cell types are listed in Table I and II respectively. Secondly, the Feulgen dye per nucleus was compared among different kinds of cells originating from one and the same animal, and the experiment of this type was repeated using rats of different body weights. The results are shown in Table III.

Since the Feulgen reaction of individual experiments was performed under different conditions, direct comparison of the data of separate experiments was not feasible. By taking appropriate tissues as the reference standard, the raw data were converted into relative values and brought together in Table IV.

Three important points were revealed by these experiments:

1. Duplicate or triplicate determinations made on the representative specimens of the same material, receiving different treatments which usually gave different numerical values of r^2E, did not affect the over-all picture of the variation in Feulgen dye content per nucleus of specimens compared. Thus, the relative values of Expt. 1 A and 1 B, of Expt. 2 A to 2 C, and of Expt. 9 A and 9 B agreed very well. Expt. 4 and 8 showed some discrepancies, but they were not serious. This suggests that the measured values are actually proportional to the DNA content per nucleus.

2. Within each of the individual animals taken at varying growth stages, the quantity of the dye per nucleus is probably a function of ploidy, regardless of the cell type. Only the class I liver nuclei of Expt. 9 showed a small deviation from the rule. Such findings are in agreement with the current theory of the approximate constancy of DNA per set of chromosomes.

3. Such a constancy rule does not hold with the cells at different stages of postnatal growth, even within the same cell types. There is a strong indication that the values of DNA per nucleus of different cells are highest in the youngest

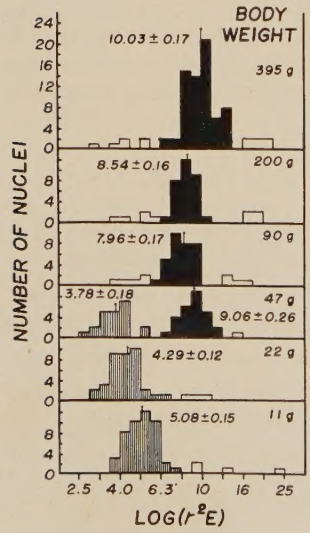

Fig. 1. Frequency histogram of r^2E (due content per nucleus) of Feulgen-stained liver cell nuclei in the course of the postnatal growth (Expt. 1 A). Shadowed and solid black parts of the histogram represent the postulated Class I and II nuclei, respectively, which were used for calculating the mean values indicated by the arrows, listed in Table I, and also given in the diagram along with the standard errors.

animals and gradually decrease until the body weight of about 50 g is reached, to remain constant thereafter or eventually to increase slowly. Different cell types showed the same trend, the liver of a rat weighing about 50 g in Expt. 2 B being rather exceptional in this respect.

As demonstrated repeatedly by previous workers [55, 53, 11, 20, 22, 44, 36], liver cell nuclei can be divided into a few classes differing in DNA content or ploidy. An example is given in Fig. 1 as the frequency histogram of Feulgen values of the individual liver cell nuclei obtained in Expt. 1 A (Table I).

TABLE I

Quantity per nucleus of the Feulgen dye developed under varying conditions: Rat livers at various stages of postnatal growth.

Experiment No.*	1 A	1 B	2 A	2 B	2 C	3
Fixation	formalin	alcohol-formalin	formalin	formalin	formalin	formalin
Reaction mixture	F	F	F	MB	B	F
Rinsing solution	A	A	B	B	B	B
Body weight range (g)	r^2E: Mean and standard error (Number of nuclei measured)**					
11–12	5.08 ± 0.15 (46)	4.09 ± 0.08 (60)	3.15 ± 0.01 (31)	3.17 ± 0.14 (32)	2.29 ± 0.06 (32)	—
22–25	4.29 ± 0.12 (37)	—	2.87 ± 0.16 (31)	2.96 ± 0.16 (28)	2.17 ± 0.09 (32)	7.43 ± 0.11 (28)
47–61	3.78 ± 0.18 (22)	2.99 ± 0.12 (29)	—	3.89 ± 0.14 (32)	—	—
	9.06 ± 0.26 (26)	—	—	—	—	*14.69 ± 0.31 (28)*
90–135	*7.96 ± 0.17 (32)*	—	*7.08 ± 0.24 (31)*	*7.13 ± 0.24 (30)*	*5.22 ± 0.17 (30)*	—
200	*8.54 ± 0.16 (31)*	—	—	—	—	—
395–400	*10.03 ± 0.17 (68)*	—	—	—	—	*16.10 ± 0.27 (20)*

* Figures pertaining to one experiment, i.e. one column, can only be compared directly with one another.

** Figures in roman and italic type represent Class I (diploid) and Class II (teraploid) nuclei respectively.

TABLE II

Quantity per nucleus of the Feulgen dye developed under varying conditions: Adrenal glands, kidney and cerebella of rats at different stages of postnatal growth.

Experiment No.*	4 A	4 B	5	6	7
Tissue	adrenal medulla	adrenal medulla	adrenal medulla	kidney cortex uriniferous tubule	cerebellum stratum granulosum
Reaction mixture	F	F	MB	F	MB
Rinsing solution	A	B	B	B	B
Body weight range (g)	r^2E: Mean and standard error (Number of nuclei measured)				
11–12	8.85 ± 0.15 (31)	9.17 ± 0.27 (30)	3.51 ± 0.10 (30)	—	4.64 ± 0.16 (28)
23–25	8.21 ± 0.16 (29)	8.81 ± 0.13 (28)	2.78 ± 0.07 (28)	8.81 ± 0.21 (28)	4.18 ± 0.15 (29)
52–61	5.93 ± 0.28 (61)	7.00 ± 0.15 (26)	1.92 ± 0.06 (30)	7.42 ± 0.17 (32)	3.44 ± 0.16 (30)
135	—	—	2.04 ± 0.17 (30)	—	3.46 ± 0.15 (30)
400	7.59 ± 0.16 (30)	7.09 ± 0.16 (29)	2.55 ± 0.14 (30)	7.97 ± 0.17 (24)	—

* Figures pertaining to one experiment, i.e. one column, can only be compared directly with one another.

The nuclei measured were selected at random, so that the illustrated diagram may be taken to represent roughly the relative frequency of nuclei of different classes. However, it may well be that the frequency of nuclei containing higher amounts of DNA is underestimated [30]. It is our experience that the peak of the histogram tended to flatten as the average values of r^2E increased. This was especially impressive when one compared measurement on separate preparations taken from one and the same material, differing significantly in the reaction intensity, as is readily realized by changing the fixative [31]. Although the exact knowledge about the relation of the magnitude of relative error to the absolute value of r^2E in our method is unavailable, such findings led to adopting the logarithmic scale of r^2E as the abscissa of the histogram.

TABLE III

Quantity per nucleus of the Feulgen dye developed under varying conditions: Comparison of different organs of rats at varying growth stages.

Experiment No.*	8 A	8 B	9 A	9 B	10
Body weight, g	12	12	135	135	150
Reaction mixture	BM	B	BM	B	F
Rinsing solution	B	B	B	B	B
Organ and cell type	r^2E: Mean and standard error (Number of nuclei measured)**				
Liver: hepatocyte	4.29 ± 0.08 (30)	3.03 ± 0.08 (31)	*8.44 ± 0.14 (33)*	*6.62 ± 0.21 (32)*	*9.94 ± 0.22 (34)*
Kidney cortex: uriniferous tubule	4.60 ± 0.11 (31)	—	4.95 ± 0.15 (33)	—	5.05 ± 0.18 (29)
Adrenal medulla	3.81 ± 0.08 (32)	2.99 ± 0.06 (31)	4.72 ± 0.13 (34)	3.61 ± 0.08 (30)	5.02 ± 0.10 (29)
Cerebellum: stratum granulosum	4.26 ± 0.07 (30)	—	4.83 ± 0.16 (29)	—	5.16 ± 0.26 (36)
Pancreas: exocrine gland	—	—	—	—	4.82 ± 0.19 (24)

* Figures pertaining to one experiment, i.e. one column, can only be compared directly with one another.

** Values for Class II nuclei are indicated in italic type.

Fig. 1 reveals that except for a rat weighing 47 g, the vast majority of the hepatocyte nuclei constitute a single prominent peak at every growth stage examined. With younger rats (11 and 22 g) this peak presumably represents Swift's Class I or diploid nuclei, and with older rats (over 90 g) it probably comprises the Class II nuclei which are assumed as tetraploid. The liver of the 47 g rat forms a transition between them, the nuclei being partitioned almost evenly into Classes I and II. It is quite impressive to see that there are only few Class II nuclei in younger, and few Class I nuclei in older rat livers and that the Class III or presumably octoploid nuclei are quite few throughout the course of the postnatal growth. As in the case with the average r^2E values reported in Table I, the peaks of the histogram representing the Class I and Class II nuclei are shifted towards lower and higher values

respectively, but the width of the peak remains largely unchanged in the course of the postnatal growth. In most cases, the overwhelming preponderance of but one nuclear class made it difficult to get the mean r^2E values with desirable accuracy for each one of the three classes in every growth stage. So, the mean r^2E was calculated only with well defined peaks and listed in Table I. More or less similar results were obtained with other specimens studied. It should be emphasized here that the division of the histogram into different peaks or nuclear classes was a matter of arbitrariness to a certain extent.

DISCUSSION

The microspectrophotometric apparatus employed in the present investigation was of improved type described by Naora [26, 28], which was constructed so as to eliminate the S-V effect [27, 28, 32] which may cause some serious error when objects with higher extinctions are to be measured. Since the extinction range of the Feulgen-stained nuclei covers such "dangerous" high values, it is not unexpected that our experiments may lead to a concept more or less different from the ones expressed previously as regards the constancy of DNA per chromosome set on the basis of inaccurate microspectrophotometry. As far as different tissue cells of one and the same animals are concerned, however, our results confirmed the constancy hypothesis supported by most of the previous investigators [1, 2, 3, 11, 20, 22, 23, 24, 39, 40, 44, 53, 54, 55, 56, 57]. Variability of DNA per nucleus in the course of postnatal growth of animals has not been a subject of systematic investigation so far, and it is in this part of our study that a discrepancy emerged between the current theory and our own findings. Accordingly, it cannot be decided that this discrepancy is actually due to the type of the apparatus used, until similar measurements are made in other laboratories with the apparatus involving the S-V effect.

Apart from the error associated with the apparatus used, there is another source of error in the determination of DNA per individual nucleus by means of the microspectrophotometry combined with Feulgen reaction. This is the quantitative reactivity of the histochemical Feulgen reaction. The problem was discussed at length by Sibatani *et al.* [47, 48, 49, 50, 52]. As recommended in their studies, measurements were repeated in the present work using separate materials and applying different fixatives or reaction mixtures to the same specimens. Inasmuch as similar results were obtained by independent observations, the measurements were assumed to be valid.

TABLE IV

Relative quantity per nucleus of the Feulgen dye developed under varying conditions. Integration of the data obtained in different experiments by taking appropriate reference standards.

Expt. No.	Organ	Body weight range, g[b]						F^d	P
		11–12	22–25	47–61	90–135	200	395–400		
1 A	Liver[a]	1.00	0.84	0.74					
				0.90	*0.79*	*0.84*	*0.99*	22.63	<0.001
1 B	Liver	1.00	—	0.73	—	—	—	65.60	<0.001
2 A	Liver	1.00	0.94	—	*1.13*	—	—	6.31	<0.01
2 B	Liver	1.00	0.97	1.26	*1.13*	—	—	8.34	<0.001
2 C	Liver	1.00	0.95	—	*1.13*	—	—	7.71	<0.001
3	Liver	—	(0.90)	(*0.89*)	—	—	(0.98)	6.43	<0.01
4 A	Adrenal gland	1.00	0.93	0.67	—	—	0.68	40.37	<0.001
4 B	Adrenal gland	1.00	0.96	0.76	—	—	0.77	33.30	<0.001
5	Adrenal gland	1.00	0.79	0.55	0.58	—	0.73	92.65	<0.001
6	Kidney	—	(0.90)	(0.76)	—	—	(0.81)	13.32	<0.001
7	Cerebellum	1.00	0.90	0.74	0.75	—	—	14.28	<0.001

	Body weight (g)	Organ[c]					F^d	P
		Liver	Kidney	Adrenal gland	Cerebellum	Pancreas		
8 A	12	1.13	1.21	1.00	1.12	—	13.78	<0.001
8 B	12	1.01	—	1.00	—	—	0.16	>0.2
9 A	135	*0.90*	1.05	1.00	1.02	—	6.11	<0.001
9 B	135	*0.91*	—	1.00	—	—	4.62	>0.01
10	150	*0.99*	1.01	1.00	1.03	0.96	1.78	>0.2

[a] Figures representing tetraploid nuclei are divided by 2 and listed in italic type.

[b] Values of 11–12 g body weight class served as reference standard except for Expts. 3 and 6, in which values of 22–25 g class were taken as standard and assumed to be 0.90. All the values calculated with the latter standard values are listed in parentheses.

[c] Values of adrenal gland served as reference standard.

[d] Variance ratio analysis to determine significance of all-over difference of mean values among individual classes (body weight groups, organs) as calculated according to Fischer.

Nevertheless, the data presented here involve a certain degree of indefiniteness. While the data of Expt. 8–10 suggest that DNA constancy rule holds with different cell types at the same growth stage or within the same individual, in Expt. 1–7 the variation in the relative value of DNA per nucleus of differ-

ent cell types at the same body weight range is a little greater than should be expected from the constancy rule (cf. Table IV). Such a discrepancy may partly be due to the experimental error, since some of the duplicate determinations with different treatments of specimens did not agree very well (Expt. 4 and 8).

Attempts to correlate the results of microspectrophotometric estimation of DNA per nucleus with those of chemical analysis have been made and moderately good agreements of the two sets of data have been reported [20, 22, 11, 44]. Of course, such attempts were made by the collective undertaking with Sibatani's laboratory. A part of the ostensible discrepancy from the DNA constancy rule, as indicated by the decrease in DNA per nucleus of livers in a very early period of postnatal growth, was also recognized by the chemical determination in his laboratory [9, 10]. The same result was obtained by Mandel [13]. However, with the material involving nuclei which belong to different ploidy classes, such as the rat liver, the comparison between the two methods is complicated, because what is obtained by the chemical analysis is the average value of DNA per nucleus, which is a function of both the amount of DNA of individual nuclei and the frequency distribution of nuclei differing in DNA content. Therefore, direct comparison of data of the other group based on biochemical determination with ours is not feasible. If the observed decrease in Sibatani's and other laboratories were due to the decreasing mitotic activity, the histogram of microspectrophotometric values at the earliest stage (11–12 g) would have to be either dimodal or so wide that it would cover both diploid and tetraploid range. Since either of these is not the case and further the variation coefficients of the two successive stages (11–12 and 22–25 g) are of the same order, it would hardly be acceptable to ascribe the change to the difference in mitotic activity. The same is true with other experiments. Davidson, Frazer and Thomson [11, 55] succeeded in overcoming the difficulty concerning the direct comparison between the chemical and microspectrophotometric methods by measuring microspectrophotometrically all sorts of nuclei selected quite at random out of the representative samples of nuclei isolated from different tissues. Microspectrophotometric measurements of the Davidson school are based on the total nuclear population of the liver, while our own data are restricted to the parenchymal nuclei. But, assuming that the non-hepatocytic nuclei of normal rat livers are all of Class I and comprise 35 per cent of the total nuclear population [10], the recalculation of the microspectrophotometric data of Thomson and Frazer [55] gives the following values: Out of parenchymal nuclei of an adult rat liver, $(58.7 - 35)/65 \times 100 = 36.5$ per cent is of Class I, $39.9/65 \times$

TABLE V

Microspectrophotometry versus gross chemical analysis. DNA per average nucleus of the rat liver tissue in postnatal growth as expressed in relative values.

Microspectrophotometry		Gross chemical analysis					
Expt. 1-A of the present study		Fukuda and Sibatani [10, 11]		Mandel *et al.* [15]		Davidson *et al.* [60]	
Body weight (g)	DNA per nucleus*	Body weight (g)	DNA per nucleus	Body weight (g)	DNA per nucleus	Body weight (g)	DNA per nucleus
11	0.95	12	0.64	14	0.87		
22	0.77	25	0.55	26	0.82		
47	1.00	50	1.00	66	1.00	35–90	1.00
90	1.17	100	1.20	129	1.12		
200	1.29	200	1.23	219	1.44	195–250	1.18
395	1.39	340	1.23	243	1.46		
				(?343)			

* Method of calculation: m = mean r^2E of all parenchymal nuclei measured. n = mean r^2E of parenchymal nuclei recognized as Class I. The mean r^2E of total nuclear population of the liver, $t = 0.65\ m + 0.35\ n$. The value of t for each growth stage is then converted to relative value.

100 = 61.4 per cent is of Class II and the rest, 2.1 per cent, is represented by the Class III nuclei. It is thus clear that in the data of the Davidson group, the frequency of Class I hepatocytic nuclei in the adult liver is much higher than that obtained in this laboratory (less than 10 per cent; see [30]). Whether this is related to the lower values of DNA per nucleus of adult rat livers obtained chemically by the Davidson group [56], or eventually due to the difference in microspectrophotometric technique employed (cf. [28, 32]) cannot be answered at the present time.

Now, the same assumption as has just been made allows us to calculate, in relative scale, the average DNA content per nucleus of total nuclear population of the liver on the basis of our photometric data of hepatocyte nuclei, the value of the rat weighing around 50 g being taken as reference standard. The results are listed in Table V together with comparable recalculations of some chemical data reported from Mandel's [13], Davidson's [56] and Sibatani's [9, 10] laboratories. It is seen that our microspectrophotometric data of Expt. 1 A agree best with the chemical data of Mandel *et al.* and Sibatani *et al.*

It may be irrelevant to compare our results directly with the ones obtained by other workers with microspectrophotometric apparatus probably involv-

ing S-V effect to varying extent. It seems, however, to be of some interest to point out certain parallels to our results in the data submitted from other laboratories concerning the maturation process of erythrocytes [14, 43], the course of development of the frog embryo [25] and of the metabolic activation of cells.

Pasteels and Lison [17, 35, 36, 37] repeatedly showed, on the basis of their microspectrophotometric studies, that the Feulgen-DNA content of the somatic nucleus is not constant, but dependent upon the physiological activity, such as mitotic activity of the cell. A part of their results were challenged by Alfert and Swift [1], but Pasteels [4, 34] continued to present new additions to the examples with which the relation they have been claiming appears to hold. The demonstration of the variation of the DNA content related with the changes of the physiological activity have been widely performed by several other groups of Belgian workers [45, 5, 6, 7, 38, 12, 59, 60]. It may not be impossible to correlate our results with their theory, because activity of mitotic division of cells declines with age, while DNA per nucleus likewise decreased during the early course of the postnatal growth in our animals. In these studies, however, ploidy classes are recognized among nuclei of one and the same organ as in the case of the present investigation and the DNA content of the individual nuclei seems to be roughly constant with a few exceptions since the variation of the histogram is almost equal in the control and experimental cases, even if the DNA content of the nucleus increases in relation to the increased physiological activity.

The pattern of ploidy partition in liver cells of adult mice illustrated by Swift [53] is markedly different from that of adult rat as shown in this paper. In mice, there remained many diploid or Class I nuclei in adults though their frequency occasionally appeared to decrease with the age of the animal. The higher frequency of Class I hepatocyte nuclei in adult rat livers in the data of the Davidson school [11] has been mentioned above. It should be recalled that the S-V effect tends to reduce the measured value of E and therefore unduly augments the frequency of Class I nuclei [28, 32]. The detailed discussion on the change in ploidy distribution in growing rat livers will be submitted as a separate paper [30].

SUMMARY

1. Microspectrophotometric determination of DNA content per nucleus was carried out with the apparatus of the Koana–Naora type, using Feulgen-stained tissue sections of rats. Usually the same set of tissue specimens

received several different treatments so as to give varying colour intensities of the Feulgen reaction, and the relative values of the Feulgen dye per nucleus were compared among different treatments. Inasmuch as separate treatments in the same experimental series gave comparable results, the dye content per nucleus was interpreted as the relative measure of the DNA content per nucleus.

2. Comparison was made between different stages of the postnatal growth of the same tissue and also between different tissues of the same individual. Apart from the complexity conditioned by the occurrence of nuclei of higher ploidy classes in the liver, the DNA content per nucleus did not vary significantly in different tissues of the same individual. But within each of the tissue examined, this value gradually decreased during earlier periods of the postnatal growth, to remain constant or slightly increase thereafter, the minimum value being reached at body weight around 50 g.

3. In young rats, most of the hepatocyte nuclei represent the diploid type, but in older rats the nuclei of tetraploid type greatly predominate. The transition can again be found around 50 g of the body weight.

The author expresses his hearty thanks to Prof. Dr. Zyun Koana, Department of Physics, University of Tokyo, for never-failing advice and encouragement in this work and to Dr. Atuhiro Sibatani, Department of Cytochemistry and Histology, Yamaguti Medical School, for valuable advice and close co-operation. Thanks are also due to Dr. Hajime Matsuda, Department of Radiology, University of Osaka, for his kind assistance and preparation of specimens, to Miss Kazuko Yosiwara and Mr. O. Harigane for technical assistance. The work was supported in part by the grant in aid for Fundamental Scientific Research as a part of the co-operate research on "Nucleic Acids".

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THE IRRADIATION OF INDIVIDUAL PARTS OF SINGLE CELLS IN TISSUE CULTURE WITH A MICROBEAM OF α-PARTICLES

I. APPARATUS

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During the past twenty years the development of micro-manipulators and micro-injection apparatus has led to an attack on the biochemical and cytological properties of single cells, and this has produced a marked advance in our knowledge of the chemical and physical processes which take place in both the resting and dividing cell. To these microphysical and microchemical processes, the ability to modify or damage single cells, or parts of single cells, by means of extremely fine beams of ultra-violet or ionising radiations has been added [1, 5–10]. It has been shown that the cytoplasm is relatively insensitive to ionising radiation but that marked changes appear in the cell when the cytoplasm is irradiated with ultraviolet light. On the other hand, the nucleus is very sensitive, small doses of either ionising or ultra-violet radiation having a profound effect on cell division. In particular Zirkle and Bloom [10] and their school in Chicago have irradiated areas of single cells circa 2 μ–3 μ in diameter with beams of fast protons, the centre of the proton beam being located with an accuracy of the order of 0.6 μ. The same school [9] has also developed apparatus capable of irradiating parts of single cells with a beam of ultra-violet light 7 μ in diameter. With the aid of this apparatus they have performed some very interesting experiments working with tissue cultures of newt heart cells. In addition, Simons [4] has described apparatus capable of producing beams of α-particles, 30 μ in diameter. As yet, no results obtained with this apparatus have been published. Up to the present the only successful work with beams less than 5 μ in diameter has been done by the Chicago school.

The present paper describes a similar, and comparatively inexpensive, piece of apparatus which is being used for irradiating single cells and parts

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of single cells of warm blooded animals in tissue culture with beams of hig energy α-particles of diameter between 1 μ and 1.5 μ.

The aim of this paper is to describe the apparatus used and to give som preliminary results of the irradiation of biological material.

Apparatus

The irradiations are carried out on the stage of a modified phase contras microscope[1] by means of a small, intense, source of highly collimated α particles which is placed between the objective and the specimen. The ce under observation can be either observed visually or photographed by tim lapse cinematography. For convenience, the description of the apparatu has been divided into the following parts: General arrangement of the appara tus; microscope stage; method of aiming the α-particle beam at the targe source of ionising radiation; and tissue culture chamber.

It is not proposed to give detailed descriptions in this paper of either th camera and electronic timing apparatus or of the method of making the coll mating holes 1 μ in diameter. These will be the subjects of separate com munications (but see p. 23–24 for a description of the collimating system

General arrangement of the apparatus.—It can be seen from Fig. 1 th the microscope is contained in a Perspex box, fitted with a heating elemer in the base and a thermostat just above the stage by which means the tem perature of the stage is maintained at $38 \pm 1°C$. The design follows th described by Hughes [2].

It has been found necessary to bring controls for three of the microscop adjustments to the outside of the perspex box. These are the fine focussin adjustment and the two adjustments required for moving the culture chamb in two horizontal directions at right angles.

The microscope is an inclined monocular type. When taking photograph an extension *A* (Fig. 1) is attached to the microscope tube, the other end which fits into the cine camera. This extension contains in addition to a shu ter, a prism which deflects the light through 90° into the eyepiece *B* whic is used for centring the cell in the field of view when the camera is in th photographing position. By means of the cable attachment the prism is move out of the light beam during filming.

In all other essentials the instrument is an ordinary phase contrast micr scope. The illumination is provided by a 250 watt high pressure mercu vapour lamp *C* (Fig. 1) in a metal housing, the light from which is filtere

[1] Manufacturers: Cooke, Troughton & Simms, Type M3000.

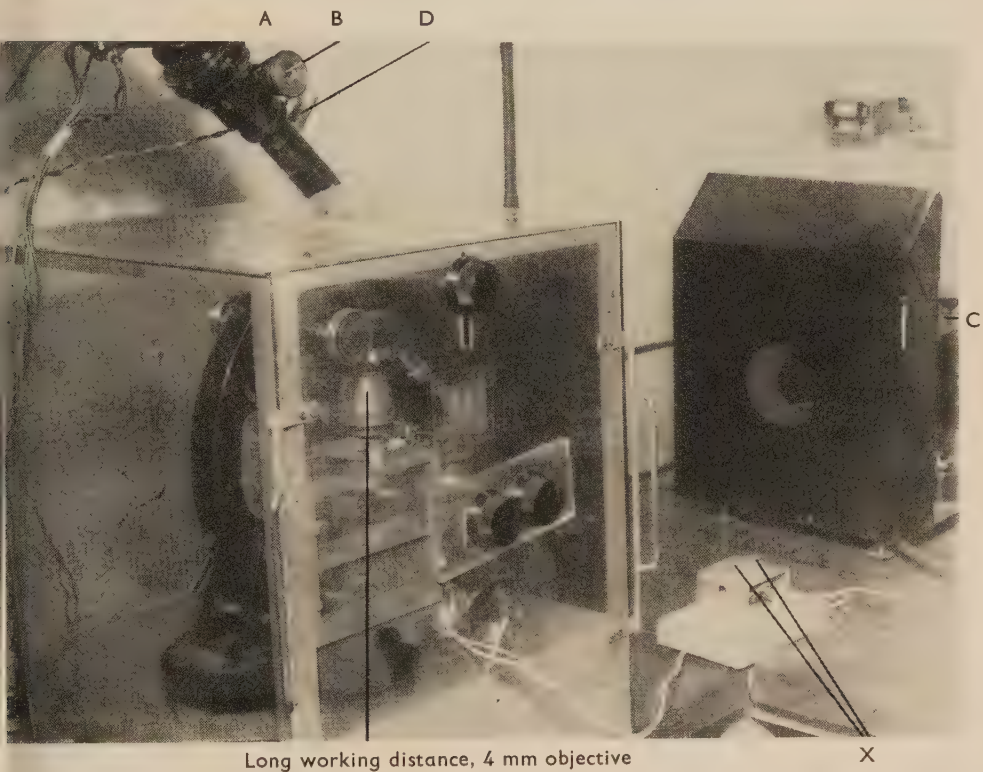


Fig. 1. General photograph of the whole apparatus.

through an infra-red filter of Chance glass type ON19 and a green filter, Kodak Type 77, which renders the emergent light effectively monochromatic of wavelength 5460 Å. The microscope is adjusted for Köhler illumination.

After irradiation, the tissue cultures are photographed either through a 1.8 mm (95×) immersion achromatic objective, or a 3.75 mm (45×) achromatic fluorite objective. Before irradiation (as will be seen later) it is essential to use an air objective, and for this purpose a Newton Long Working Distance attachment fitted with a Cooke Troughton & Simms 4 mm phase contrast objective, is used. The working distance is thus increased from the normal 0.70 mm for a Cooke 4 mm objective to 12.8 mm. The Newton Long Working Distance Attachment is normally supplied fitted with a Beck 4 mm objective. It was adapted, however, for use with the Cooke, Troughton and Simms 4 mm phase contrast objective and Mr. P. C. Smethurst[1] kindly made a special

[1] of Smethurst High Light Ltd., Bolton, Lancs.

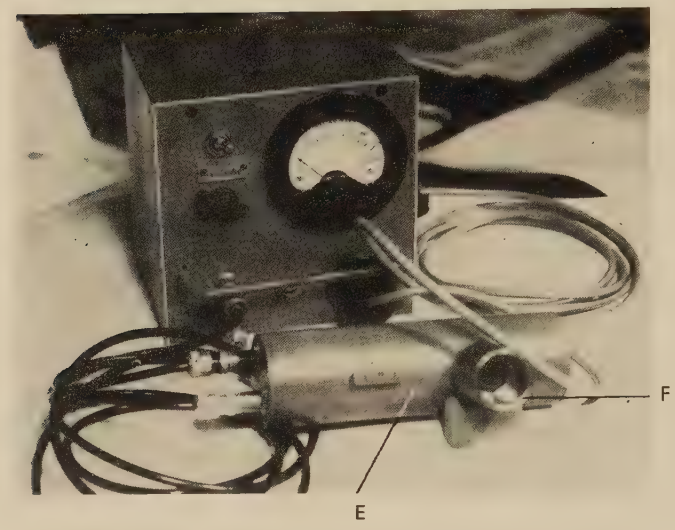


Fig. 2. Photograph of in strument for measuring in tensity of the microscop image.

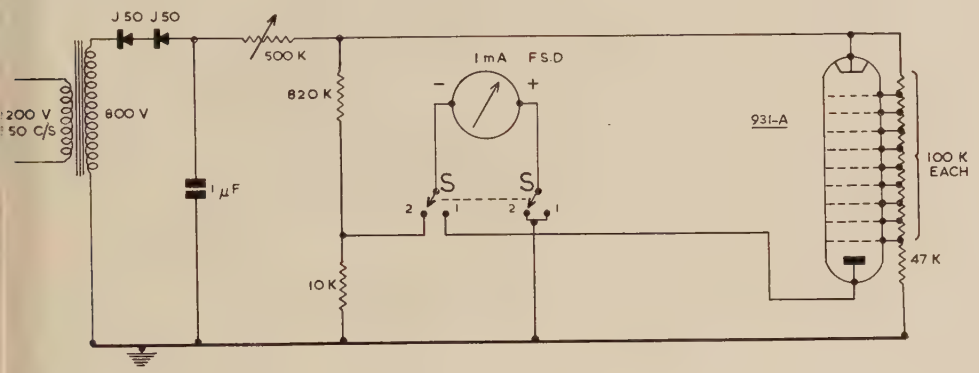
condenser phase annulus to our specifications; this is essential when th 4 mm Cooke phase contrast objective is mounted in the Long Working Dis tance Attachment.

The eyepiece is fitted with crosswires which are necessary for aligning th α-particle beam on the target. During alignment (*vide infra*) the field o view is completely dark and the crosswires are invisible. In order to rende them visible they must be illuminated, and for this purpose the microscop tube is fitted with a brass side tube *D* (Fig. 1) into which is inserted a 3 volt flash lamp bulb. A slit in the side of the eyepiece tube allows the ligh from the bulb to illuminate the edge of the circular glass graticule on whic the crosswires are engraved. Adequate illumination is obtained by supplyin the 3 volt bulb from a small 1.5 volt dry cell.

To obtain good photographs, it was essential to be able to measure the bright ness of the image and also to be able to change the brightness by means o neutral absorbers. For this purpose a photo-electric light intensity meter ha been designed and constructed (Fig. 2).

A brass tube *E* contains a photomultiplier, Type 931A. The tube *E* ha a sidearm *F*, one end of which is open and fits over the ocular end of th microscope tube, thus permitting the cathode of the photo-multiplier to b illuminated by the light emerging from the microscope. The electrical circu of the device is shown in Text-fig. 1. When the double-pole, double-thro switch *S* is in position 1, the 0–1 milliameter measures directly the outpu from the anode of the photomultiplier and hence the light intensity. In pos

tion 2 the switch converts the milliameter into a voltmeter measuring the voltage across the photomultiplier which may then be set, by means of the variable 500,000 ohm resistance, at some pre-determined standard voltage. By this means the light intensity meter is adjusted every time before use to have approximately the same sensitivity.



Text-fig. 1. Circuit diagram of image intensity meter.

The ciné-camera takes 16 mm film and consists of a modified Royal Air Force camera, type G45, obtained from Government surplus stores. The mechanism was modified and the camera drive is controlled by the electronic time lapse apparatus (to be described in detail elsewhere). By this means the time interval between exposures can be varied from 2 seconds to 16 seconds. The exposure time, however, remains constant, whatever the value of the time interval between exposures. An auxiliary eye-piece fitted to the camera, permits the image in the plane of the photographic frame to be viewed and focussed correctly during photography.

Microscope stage—method of adjustment.—The main modifications and additions to the microscope were made on the stage, a photograph of which is shown in Fig. 3 and a diagram in Text-fig. 2.

The normal stage consists of two plates, of which the lower is attached rigidly to the body of the microscope and the upper slides above the lower one in guides. This allows movement of the specimen in one direction. Movement in a direction at right angles is achieved by moving the specimen holder in guides with respect to the upper plate.

In order that the α-particle source shall move independently of the upper plate of the stage which carries the specimen, the source is supported at the end of an arm *G* firmly attached to the lower plate by the solid steel post *H*. This post passes through the upper plate in which a hole is drilled large

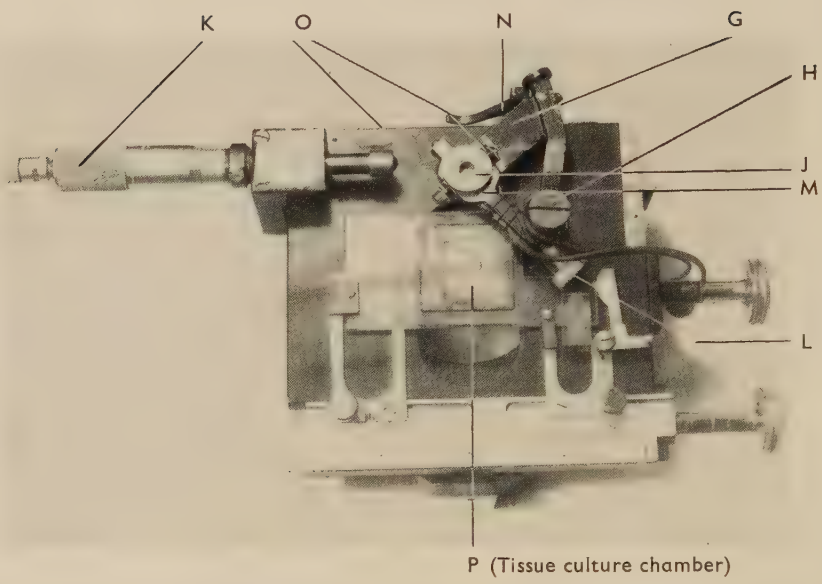


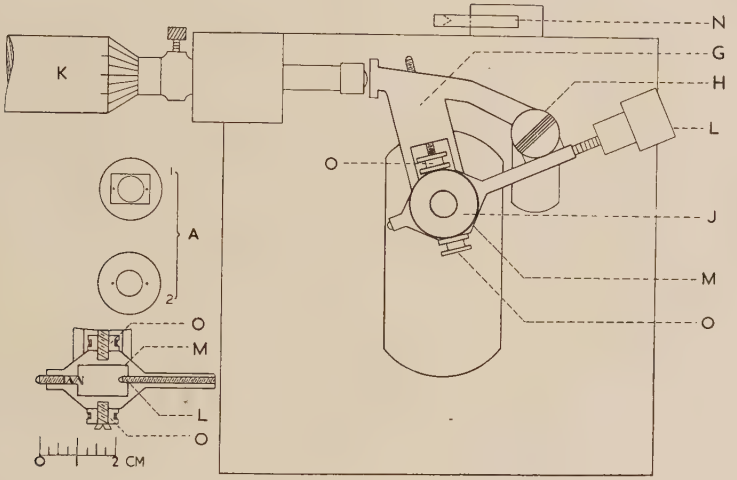
Fig. 3. Photograph of microscope stage.

enough to allow sufficient relative movement between the two plates to enable a convenient area of the tissue culture to be searched. The irradiation is from above and the source is situated in a hole *J* at the end of the arm *G*. The source may be moved relative to the tissue culture in two directions. Adjustment of the micrometer screw gauge *K* produces movement of the source along the arc of a circle and adjustment of the screw *L* moves the source holder within the guide *M* in a direction approximately perpendicular to that produced by the micrometer screw. By this means the position of the source may be adjusted so that the image of the hole used for collimating the α-particles may be brought into coincidence with the image of the cross point of the hairs fitted into the eyepiece. This adjustment can be made with an accuracy of about $\pm 1\ \mu$.

From the previous description it will be realised that although the microscope controls were obviously adjusted to have as little backlash as possible, the presence of backlash does not affect the accuracy of the aligning adjustments, but merely makes them more difficult.

Source of ionising radiation.—Several possible sources of ionising radiation are available, viz., α-particles, β-particles, accelerated electrons, γ-rays, X-rays, fast protons and deuterons. It was concluded that, although other sources could be used, an α-particle emitter provided the simplest and most convenient source for the following reasons.

Fast protons and deuterons were immediately ruled out as there are no natural radioactive sources of these particles; an accelerator would be expensive and cumbersome and would make it difficult to arrange for the irradiations to be carried out on the stage of the microscope. A source of γ-rays, which can be obtained from many natural radioactive elements, was rejected because the ranges of the secondary electrons in tissue, produced by absorp-



Text-fig. 2. Diagram of microscope stage.

tion of the γ-rays, would have ranges much larger than the diameter, 1–2 μ, of the collimated beam it was desired to produce. For example, γ-rays of 500 keV energy produce secondary electrons of a mean energy of 170 keV and these electrons have a range in tissue of 300 μ.

Nor are accelerated electrons a suitable ionising agent, since, because of the absorbers they must necessarily pass through, their energy must be at least 45 keV and such electrons produce secondaries which have a maximum range in tissue of 10 μ. When we consider soft X-rays it can be seen from Table I that for quantum energies between 5 keV and 10 keV the ranges of the secondary electrons produced are reduced to the same order of magnitude as the diameter of the collimating hole. At 5 keV a gold collimator 0.02 mm thick suffices to reduce the intensity outside the beam to 1 per cent of that in the beam.

Assuming that the minimum distance between the X-ray source and the tissue culture might be about 1 cm, it is possible to calculate the required output from the tube. From Text-fig. 3 it can be seen that the effective area of

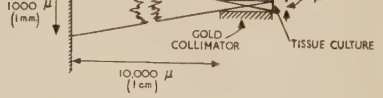
TABLE I

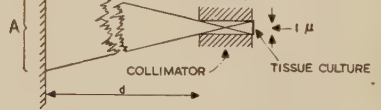
X-ray energy keV	Mean electron energy keV	Range of electrons in tissue of unit density. μ
20	15	5.1
10	10	2.5
5	5	0.75

the X-ray source from which radiation may reach the culture is 1 mm², and if it is assumed that the intensity needed should be sufficient to produce 1 ion pair/μ^3/sec. in the target, i.e., ca. 0.6 r/sec., then 1 mm² of the X-ray tube anode surface must produce about 40 r/min. at 1 cm. This is just the intensity which can be obtained from a commercial tube having an anode of area 1 mm². Soft X-rays are therefore a possible ionisation source for micro-beam experiments.

There remain α-particles. From Text-fig. 4 it can be seen that the required specific activity of the source (activity/cm²) must be, to a first approximation, independent of the distance, d, between the source and collimator, since the area A of the source from which particles may reach the target is approximately proportional to d^2 and the intensity of the radiation produced by any small area of the source at the target is approximately proportional to $1/d^2$. These two factors cancel and for a given specific activity the intensity at the target is independent of d provided that the source area is greater than A. Assuming that about 10 α-particles per min. passing through an area of 1 μ^2 of the target is the order of radiation intensity required a simple calculation shows that a specific source activity of ca. 6 mc/mm² is necessary.

This specific activity is readily and easily available from natural radioactive sources, e.g. polonium, which also possesses the further advantages of (1) being practically a pure α-emitter, (2) being inexpensive, and (3) having a reasonably long half-life (138 days). The energy of the α-particles emitted





Text-fig. 3 (*left*). Geometro of X-ray source and irradiated tissue culture.
Text-fig. 4 (*right*). Geometry of α-particle source and irradiated tissue culture.

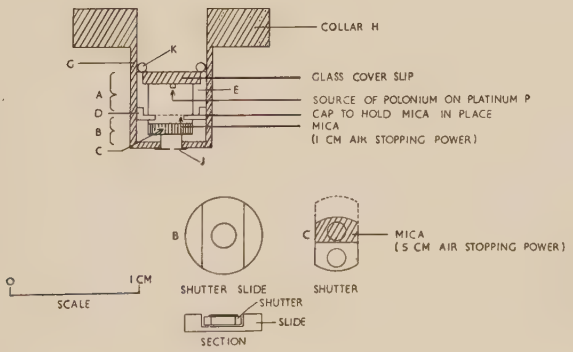
TABLE II

Metal	Z	Thickness of metal needed to stop α-particles. μ
Al	13	12.8
Cu	29	5.5
Ag	47	6.1
Au	79	4.6

:5 MeV with an extrapolated range in air of 3.9 cm. It is necessary for these rticles to pass through two layers of absorber (see later) and 0.5 cm air be- e entering the tissue. In practice this leaves a residual range of ca. 1.4 cm air or 10 μ in tissue when the particles enter the tissue cells. This is ade- ate for the purpose desired. For collimation, only thin films of metal are eded as can be seen from the list giving the ranges in several metals of α- rticles having a range of 2.4 cm in air corresponding to the energy they ve when striking the collimator.

For the reasons outlined above, we have chosen a collimated source of particles from ^{210}Polonium for the present experiments, although it appears at soft X-rays might also have been used.

Text-fig. 5 is a diagram of the source and collimating system. The polonium urce was supplied by the Radiochemical Centre, Amersham, to our speci- ations and initially consisted (in July 1955) of 8 mc of pure polonium de- sited on a piece of thin platinum foil 1 mm square. The platinum foil *P* e Text-fig. 5) is attached, with a minute quantity of Durofix, to the glass verslip which is itself also stuck with Durofix on to the brass tube *E*.



t-fig. 5. Diagram of α-particle rce and collimating system.

A mica window, of stopping power 1 cm air equivalent, attached to a brass cap *D* closes the source container *A*. If it is desired to dismantle the source the cap *D* can be pushed off. On the other hand such manipulation should be avoided as far as possible since polonium is an extremely toxic substance.[1] The α-particles emerge through the mica window with a range of 2.3 cm in air. The source and holder *A* are inserted into a brass tube *G* fitted with a wide collar *H* having a knurled edge for easy manipulation. Beneath the source container is a small steel shutter *C* which moves in a brass guide *B*. There are two holes in the shutter and the movement allows either of the holes to be brought into the centre. One hole is covered with mica of 5 cm air equivalent stopping power and the other is uncovered. In one position the α-particles can impinge on the collimator *J* and in the other position they are prevented, by the mica, from so doing. A split ring *K* holds the whole assembly in place.

The collimator *J* is attached to the end of the tube and consists of four copper discs each 3 mm in diameter and 20 μ thick. The upper three have central holes 100 μ in diameter and are stuck together with Durofix; the bottom one has a central hole 1–1.5 μ in diameter and is stuck to the others by means of shellac. The detailed method of making these holes will be published elsewhere.

It was found necessary to include the triple thickness subsidiary collimator with 100 μ hole, to stop most of the disintegration protons arising from the reaction

$$^{14}_{7}\mathrm{N} + ^{4}_{2}\mathrm{He} \rightarrow ^{17}_{8}\mathrm{O} + ^{1}_{1}\mathrm{H}$$

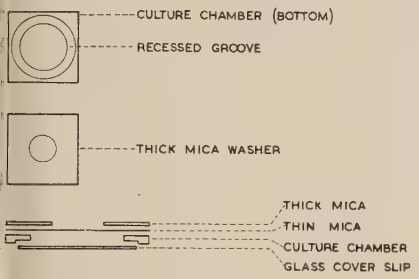
produced by the intense bombardment of the small quantity of air enclosed in the source container. These protons which have a range of 29 cm in air were found to be very troublesome in the early stages of the work.

When in use the whole assembly of source, shutter and collimator is inserted into the hole *J* (Text-fig. 2) of the swinging arm (Fig. 3) and rests gently under its own weight, on the mica coverslip of the tissue culture chamber. The whole source assembly is effectively transparent and when in position and illuminated from below, a magnified image (and diffracted pattern) of the collimating hole can be clearly seen in the microscope eyepiece. The dimensions of the holes in the shutter and various apertures have been designed so as to retain the full numerical aperture of the 4 mm objective

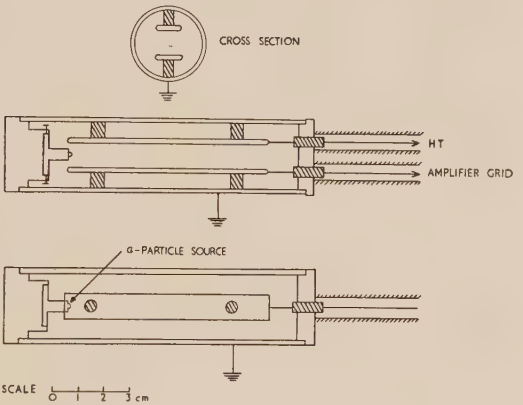
[1] Although polonium is well known to be a mobile element, able to diffuse rapidly across metal surfaces, this type of source holder has been found quite satisfactory in use and not to become active on the outside.

and Long Working Distance Attachment; the only effect of the platinum disc (on which the polonium is deposited) is to obstruct a small area in the centre of the field and reduce the intensity of illumination by about 10 per cent.

The shutter *C* (Text-fig. 5) is activated electromagnetically by means of the two small coils *O* (Text-fig. 2 and Fig. 3) with iron cores which are inset into



Text-fig. 6 (*left*). Design of tissue culture chamber.



Text-fig. 7 (*right*). Diagram of α-particle ionisation chamber.

the fixed brass support of the slide holding the source. The coils which are supplied by Amplivox Ltd have 1029 turns and 200 ohms resistance; they are wound on a polythene former of outside diameter 6.5 mm and 3 mm deep with a central hole 3 mm in diameter through which the iron core fits. A short pulse of 250–300 ma current is sufficient to activate the shutter and the two switches *X* (Fig. 1) are used for the shutter control.

Tissue culture chamber.—The biological material used for irradiation is prepared as a hanging drop tissue culture in a specially designed chamber (Text-fig. 6). The main part of the chamber is made of brass which is rhodium plated after fabrication; the lower side is closed by means of a glass coverslip 4 mm in diameter which is inset slightly into the brass and sealed with paraffin wax. The upper side of the chamber is closed by a square piece of mica. On top of a piece of mica 2.5 cm square and of 1 cm air equivalent stopping power, is placed a sheet of thicker mica also 2.5 cm square with a centrally located hole of 1 cm diameter. The two layers of mica, which are held together with a paraffin vaseline mixture, rest on top of the brass body of the chamber and the brass–mica junction is sealed and made air-tight with the paraffin–vaseline mixture. A corner of the brass part of the

culture chamber holder is cut away to prevent contact with the screw G (Fig. 3) when the culture chamber is moved while searching for a suitable cell.

Dosimetry

From the geometry of the α-particle source and collimator it is evident (Text-fig. 4) that the beam of α-particles should have an angular spread of half width 3°. Thus a beam which has a diameter of 1 μ on emerging from the collimator will have a diameter of 2 μ at a distance of 10 μ from the collimator. Before entering the culture, the beam must traverse a sheet of mica 5 μ thick and it is evident that in order to retain a narrow beam the closest possible contact must be achieved between the collimating hole and the mica supporting the culture.

The first measurements of the beam diameter were made by placing the source and collimator hole in contact with Ilford C2 nuclear photographic emulsions 50 μ thick. After developing, the resulting patch of α-particles varied in diameter from 3 μ to 15 μ for a given hole and constant exposure time; it was evident that the contact between the plate and collimating hole was not good, owing to there being a small angle between the lower surface of the collimator and the emulsion. The thin mica film supporting the culture will, however, bend and conform to the angle of the plane of the collimator.

It was therefore decided to make the measurements under exactly similar conditions to those which would exist during the experiment. A piece of Kodak stripping film of the type used for autoradiographic work with electrons, was mounted with the emulsion side towards a piece of mica of 1 cm air equivalent stopping power which had itself been mounted over a rectangular slot, 1 cm × 2.5 cm, cut into a perspex slide about the size of a microscope slide. The emulsion of the film is 4 μ thick and is backed by a layer of gelatine 10 μ thick. Exposures of the plate to the α-particle source were made on the microscope stage in the same way as an irradiation would be carried out during an experiment on a culture. Exposures of varying duration were made in a series along the length of the plate, spaced at intervals of about 0.25 mm. Fig. 4 shows photomicrographs of exposures made on C2 emulsions and also on stripping film under operating conditions. The stripping film was viewed under the microscope from the gelatine side, and hence the wider end of the spreading α-particle beam was measured. It can be seen from these photographs that the diameter of the beam is about 1.5 μ when collimated by a hole which appears optically to be 1 μ or less in dia-

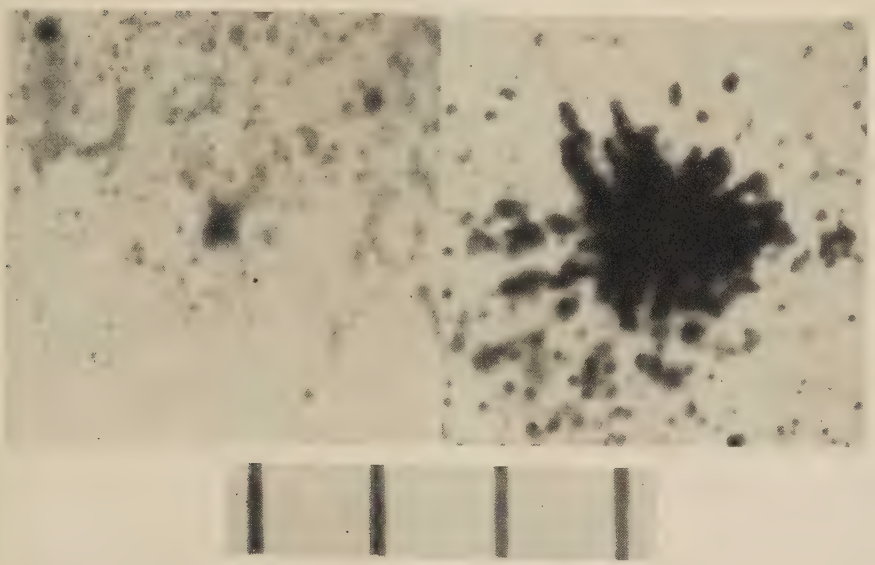


Fig. 4. Photomicrographs showing diameter of α-particle micro-beam. *Left*, exposure on stripping film. *Right*, exposure on C2 nuclear emulsion plates (emulsion 50 μ thick). Scale for both photographs 1 division = 10 μ.

meter. The exposure time was 15 seconds which is of the same order as the exposure given in the biological experiments.

For the purposes of the biological work it was also necessary to know the rate of emission of α-particles through the hole and their energy distribution on emerging. This was easily determined by placing the source very close to the edge of an Ilford C2 emulsion nuclear photographic plate (Fig. 5) and allowing the particles to enter at a very small angle ($\sim 3°$) to the emulsion. From measurements made under the microscope on the emulsion, after development and fixing, a numbers-energy curve was constructed (Text-fig. 8).

This was not, however, a practicable method for daily counting because of the delay between the exposure and the track counting and measurement. An ionisation chamber (Text-fig. 7) and linear amplifier (gain $\sim 10^5$) were therefore constructed and counts of the α-particles emerging from a hole were made, a standard Type 1009A scale of ten counter being used at different discriminator bias voltages. Comparison of this curve with that obtained from the nuclear plate measurements allowed us to calibrate the discriminator bias voltage in terms of absolute α-particle energy. Two such curves, plotted so that the end points coincide, are shown in Text-fig. 8. It can be seen that the shape of the curves giving the distribution of the particles

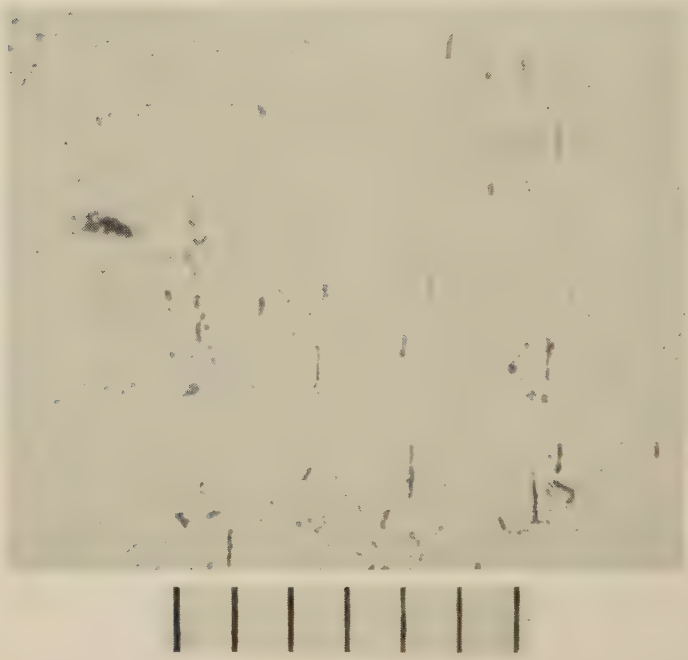
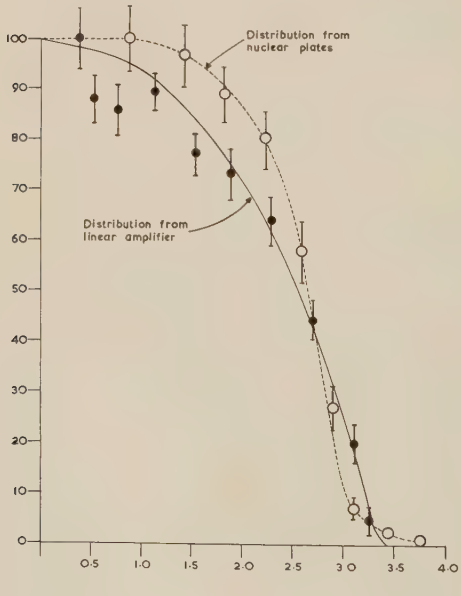


Fig. 5. Beam of α-particles emerging from collimator and entering surface of C2 emulsion at sma
angle. 1 division = 10 μ.



Text-fig. 8. Numbers-energy curves of emerger
α-particles.

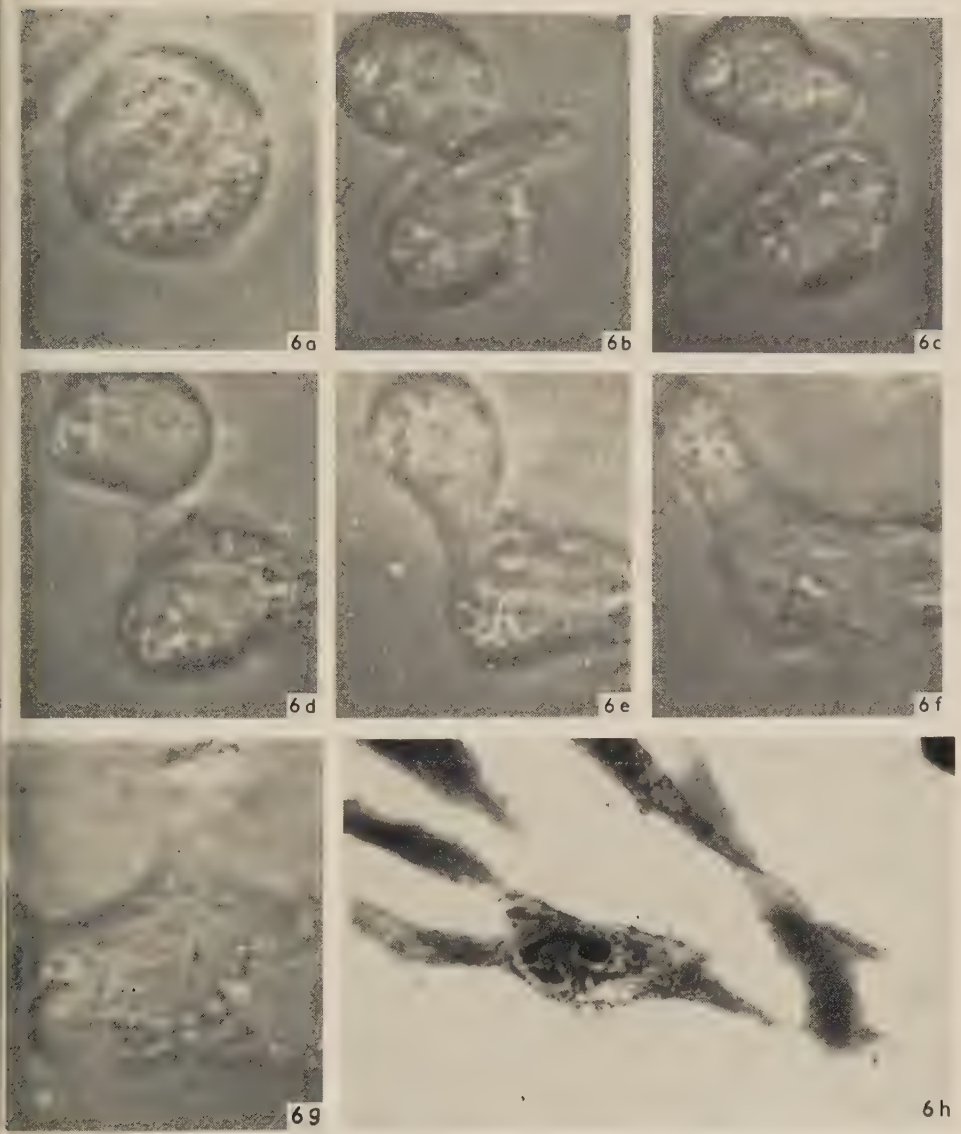
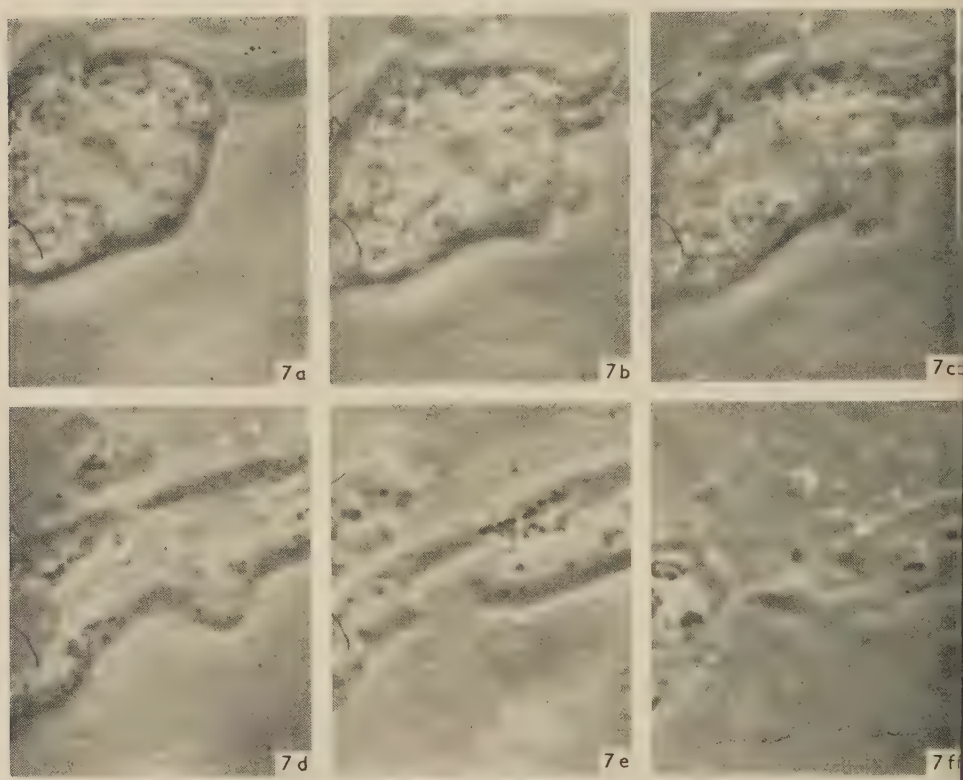


Fig. 6. Metaphase plate irradiated with 4 alpha particles in a beam 1 micron in diameter. Phase contrast photomicrographs. Magnification × 1750. (*a*) Cell immediately after irradiation; (*b*) cleavage after 1 hour 15 minutes in metaphase; (*c*) two cells separate; (*d*) threads seen connecting two cells; lower cell seems to contain most of chromosome material; (*e*) re-fusion of daughter cells 12 minutes after (*c*); (*f*) nuclear division occurs; (*g*) no cytoplasmic division; binucleate cell forms 1 hour 45 minutes after irradiation.
Stained: Heidenhain's haematoxylin. Magnification · 1100. (*h*) binucleate cell with clumped chromosomes.



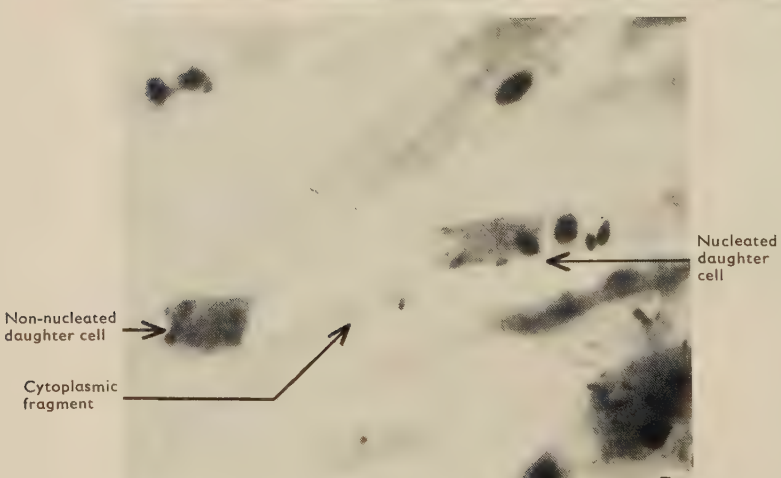


Fig. 7. Spindle fibre irradiated with 45 alpha particles in a beam 1 micron in diameter. Phase contrast photomicrographs. Magnification × 1750. (*a*) Chromosomes plainly visible on metaphase plate immediately after irradiation; (*b*) cell lengthened 4 minutes after (*a*); chromosomes do not pull apart; (*c*) 2 minutes later; (*d*) piece of cytoplasm separated off; cell continues to pull apart; 7 minutes later; (*e*) 10 minutes later; (*f*) cytoplasmic division but no nuclear division has taken place in 30 minutes after irradiation.

Stained: Heidenhain's haematoxylin. Magnification × 1400. (*g*) non-nucleated fragment seen on the left; cytoplasmic strand lies between daughter cell and cell with single nucleus is on the right

s the same. Thus, for day to day measurements of the α-particle energy istributions, the ionisation chamber and linear amplifier were used and hecks were done at longer intervals by comparing measurements made vith the nuclear plates and the ionisation chamber.

Modus operandi

The final design of the irradiation apparatus with the Long Working)istance Objective and transparent source was evolved only after several nsuccessful designs had been tried and discarded. The success of the final esign lies in the fact that the only microscope adjustment necessary during n irradiation is to raise and lower the objective with the fine focus adjustment, by a small amount of the order of 5–6 μ. Microscopes made by several manufacturers were tested, all of which when so adjusted return the optical xes of the objectives to well within 1 μ laterally of their original position. Even the most careful movement of the coarse focussing adjustment, however, produces horizontal changes in position of the order of 20 μ; it is essential that in any microbeam work in which the irradiation is to be given on the microscope stage, no adjustments should be made during the alignment or irradiation, requiring movement of any of the microscope components other than that produced by the fine focus.

The method of making the irradiation is quite simple. First, the position of the small hole, with the swinging arm in position for an irradiation, is adjusted, by means of the micrometer screw *K* and adjustment screw *L* Fig. 3) so that it is approximately in the centre of the field of view when viewed with the Long Working Distance Objective. The source and collimator are now moved away, and held back by the hook *N*. The tissue culture chamber *P* is placed in the slide holder on the microscope stage. The tissue culture is now searched until a suitable cell for irradiation is found. The image of that part of the cell which it is desired to irradiate is then aligned with the centre of the crosswires in the eyepiece, and the source and collimator are swung into position above the culture chamber. It has been found that though the hole does not return exactly into the centre of the field of view, its position does not change by more than 10 μ. When the source is moved into place, it is raised by a two pronged brass fork 1 mm thick, which is inserted under the collar (Text-fig. 4) of the source holder; thus the collimator is prevented from becoming blocked or damaged by scraping the paraffin on the top of the culture chamber. When the source holder is in position, it is released by gently removing the fork and so allowed to come

to rest under its own weight on the thin mica on which the culture is grow
The fine focussing adjustment of the microscope is now used to raise
objective until the hole comes into focus and can be seen as a bright spo
the field of view. The rest of the field is completely dark and so the eyep
crosswire illumination is now switched on. By means of the microm
K and adjustment screw *L* (Fig. 3) the hole is moved until its image coinc
with the centre of the crosswires which have previously been aligned
that part of the cell which is to be irradiated. The whole of this alignme
made with the shutter of the source in the off-position so that no α-parti
enter the culture. The shutter is now moved and the irradiation begins.
is continued for the desired time. At the end the shutter is closed, the so
is carefully raised by means of the fork and the whole shutter assen
is moved back until held again by the hook *N* (Fig. 3). By lowering the ob
tive with the fine focussing adjustment only, the culture is again bro
into focus to see if the irradiated cell has moved during the exposure. I
cell has not moved and the irradiation has therefore been satisfactory,
Long Working Distance Objective is changed for the oil immersion 4
Achromatic Fluorite Objective and filming is begun.

EXAMPLES OF RESULTS

Some specimen photographs of cell abnormalities produced by
irradiations are shown in Figs. 6 and 7. In these pictures tissue culture
chick fibroblasts were used.

Example I (Fig. 6).—An α-particle beam 1 μ in diameter was aime
the metaphase plate of a cell. Energy distribution curves show that a
4 alpha particles had sufficient energy to enter the cell.

The cell is shown two minutes after irradiation in (*a*); its contents
seen to move actively and it also wandered about in the culture. The
remained in metaphase for one hour and fifteen minutes. At the end of
time, with no unusual bubbling, the cytoplasm divided (*b*) to form
daughter cells. During the filming it appeared as if one cell contained
a few chromosome fragments, and most of the chromosome material see
to have remained clumped in the other daughter cell. The two cells
apart (*c*), joined by only a thin thread. As the cells separated further, se
connecting threads appeared in focus at various times (*d*). The two cells f
(*e*) 87 minutes after irradiation, and in (*f*) the cell entered what appe
to be an anaphase which was never completed. A single cell was fo

f) which contained clumped chromosomes, visible in the stained preparation (*h*) fixed 2 minutes afterwards.

Example II. (Fig. 7).—An α-particle beam 1 μ diameter was aimed at the spindle area of a metaphase cell; 45 particles had sufficient energy to enter the cell.

Since the spindle is very short in chick fibroblasts, it is difficult to hit the spindle and at the same time miss the chromosomes, unless the beam is very well collimated and accurately aimed. An extremely large dose of alpha particles was given to the spindle area since it was expected that the spindle would be highly resistant to the radiation. Such a large dose would produce a drastic effect on the cell if the chromosomes had been hit. The spindle is not easily visible in the phase contrast microscope. From observations made with a polarizing microscope, Hughes [3] has estimated that the spindle of chick fibroblasts is about 10 microns in length at metaphase, extending to 14 microns in anaphase. This means that the pole is only 5 microns from the chromosomes during metaphase. If the average cell in metaphase is about 20 microns in diameter, the pole must also be about 5 microns from the cell membrane. One may hope, therefore, to hit the spindle if one aims at a point 3 or 4 microns from the metaphase plate. If, after the irradiation, the alignment cross hairs still intersect at the same point in the cell and the chromosomes have not moved, then it may be reasonably assumed that the spindle area only has been irradiated. This was achieved in two out of six attempts. The results of these irradiations were so strikingly different from all others in which either the metaphase plate or a more remote portion of the cytoplasm were irradiated, that it seems probable that the spindle was hit and damaged.

The cell is seen two minutes after irradiation in (*a*), showing the chromosomes clearly arranged on the metaphase plate. Some cytoplasmic bubbling was already visible; it became more pronounced and the cell elongated although there was no anaphase movement of the chromosomes (*b*) and (*c*). As the cell elongated further a bleb of cytoplasm was budded off (*d*); and the chromosomes were no longer visible. The two parts of the cell drew further apart (*e*), and separated completely within a half hour after the irradiation (*f*). In the stained culture (*g*), fixed 10 minutes later, a non-nucleated cytoplasmic fragment is seen connected by streaks of free cytoplasm to a cell containing one nucleus. The two dark staining particles lying at the edge of the cell are possible accessory nucleoli. The nucleus did not divide.

SUMMARY

A method of irradiating parts of single cells in tissue culture on a mic scope stage with beams of α-particles 1 μ–1.5 μ in diameter is described w the necessary ancillary apparatus. Two examples are given of cell abn malities produced by such irradiations as series of phase contrast photomic graphs taken from a ciné film and photomicrographs of the stained cultu at the end.

We gratefully acknowledge the skilled technical assistance of Mr. P. Daniels, help of Mr. P. C. Smethurst in supplying us with copper discs with holes 5 μ or 6 μ diameter from which the collimators were prepared, the untiring efforts of M Simon-Reuss who has prepared all the tissue cultures and done much developm work testing materials for making the culture chambers, and the unfailing encoura ment of the Director of the Laboratory, Professor J. S. Mitchell.

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THE GROWTH PATTERN OF HUMAN CANCER CELLS IN TISSUE CULTURE WITH *MYCOBACTERIUM TUBERCULOSIS*

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LTHOUGH investigators have been interested for many years in the relaonship between the tubercle bacillus and the host cell, there does not seem be an agreement concerning symbiosis and toxic effects in tissue culture. he results presented here concern a study of human epidermoid carcinoma ssue cultures infected with concentrated and dilute suspensions of virulent *lycobacterium tuberculosis* var. *hominis*, over a prolonged six-month period ithout transfer.

Fischer [8] reported symbiotic growth for a year in tissue culture when vian tumour cells became infected through tuberculous chicken plasma. oth cells and bacilli survived in good condition through numerous transfers. An injurious effect on tissue cultures from a susceptible host was described r the tubercle bacillus in reports of studies by Maximow [16, 17], Haagen 4], Timofejewsky and Benevolenskaja [23]. Smith, Willis and Lewis [21] und no degenerative changes in embryonic chick tissues grown together ith tubercle bacilli for a few days.

An extensive study of the problem was undertaken by Brieger [4], Fell nd Brieger [7], and Brieger, Fell, and Smith [5]. They reported no toxic ffect, but a destruction of cells in tissue cultures from infected animals, and n tissue cultures infected *in vitro*. They noted that cells in excellent condition uld be seen in close association with masses of tubercle bacilli. They agreed ith Lurie [15] that living cells promoted rather than inhibited the growth f tubercle bacilli in tissue culture. Barski [2] also noted that cells and tubercle acilli grew well together. Shepard [20] reported an increased phagocytosis of uman virulent tubercle bacilli by HeLa cells when the medium contained orse serum.

MATERIALS AND METHODS

A series of experiments was designed to study infection in general at the cellular vel, and in particular the effect of the tubercle bacillus and substances associated

with tuberculosis. Because of the chronic nature of the tuberculous process, it seeme that a hardy type of cell was most desirable for this purpose, a tissue culture stra which was stabilized and able to remain viable for long periods of growth with th tubercle bacillus, without necessitating media renewal and transfer.

A stable uniform strain of human epidermoid carcinoma cells, Af (H. Ep. No. had been developed [10, 11, 24] and was used for the studies presented. The grow rate of this strain had been studied [13]. Cultural characteristics were uniform. T cells have been maintained in tissue culture since September 1952.

A streptomycin resistent virulent strain (R 6157) of human tubercle bacilli w obtained from Dr. H. Engbæk of the State Serum Institute, Copenhagen, for use two experiments, which were done with eleven-day old Dubos tween albumin cultur The cultures were centrifuged for 10 minutes at 3000 rpm, the sedimented bac washed three times in Gey's balanced salt solution,[1] and suspended in Gey's tiss culture medium.[2] The medium containing bacteria was used as the fluid phase the experimental cultures. Bacterial counts were done according to the oleic a agar plate method [19]. The concentrated suspensions contained 45,000,000 infecti units and were diluted, so that concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 1 were obtained. Since the bacteria tend to clump, it is possible that a higher dilut contained a larger number of individual tubercle bacilli than a lower dilution. T number of tubercle bacilli was estimated in terms of infective units.

The human serum and horse serum used in the suspension both gave low titre the Middlebrook–Dubos haemagglutination test [18]. The human serum donor h a natural positive Mantoux skin test.

During the cultivation period the cultures were maintained in Porter flasks a chick plasma–chick embryo extract clot. One ml Gey's medium was used as fluid phase.

A tissue culture technique was devised, based on work done by Ebeling making possible growth measurements and histological studies without disturb the experimental cultures over a prolonged period of cultivation. All tissue cultu used were derived from subtransfers from a single mother culture, and were of same age and condition. Three tissue cultures approximately one cm^2 in size w used for each set of experiments. The cultures were quartered diagonally, and dist uted in four Porter flasks, so that each flask had one quarter of each of the t cultures. One culture in each flask was placed on a coverslip. One flask served as control, and the remaining three as experiments. The bottoms of the flasks w coated with 4 drops of chick plasma, and the sections were placed in the flasks w 2 drops of chick embryo extract.

Using a photographic projection apparatus, the outline of each culture was dra and the area measured by means of a planimeter at the beginning of the experime and at intervals during the period of cultivation. Each value in the tables repres the average growth for 6 flasks, or two sets of experiments.

Growth was expressed in growth index values in accordance with Ebeling's t nique [6]:

[1] Gey's balanced salt solution: NaCl, 8 g; KCl, 0.375 g; $NaHCO_3$, 0.25 g; $CaCl_2$ (anhyd.), 0 $MgCl_2 \cdot 6H_2O$, 0.21 g; $Na_2HPO_4 \cdot 2H_2O$, 0.15 g; $Na(K)H_2PO_4$, 0.25 g; Glucose, 2.0 g per liter.

[2] Gey's medium: 5 parts Gey's balanced salt solution, 2 parts chick embryo extract, 1 horse serum, 2 parts human serum, 25 units penicillin and 25 micrograms streptomycin/m

$$\frac{\text{Total tissue culture area per flask minus original total tissue culture area per flask (experiment)}}{\text{Total tissue culture area per flask minus original total tissue culture area per flask (control)}} = \text{GROWTH INDEX}$$

Thus a growth index less than one indicated depression of growth compared with the control, and a growth index of more than one indicated stimulation. The uniform growth, the even edge of the cultures, and the ease with which they were removed from the clot made possible the use of this system. Using untreated "experiments" in groups of 5, normal values ranged from 0.80–1.21 [13].

The cultures could be examined daily, and from time to time tissue could be removed for histological study. When removed and stained *in toto*, those cultures planted on coverslips in the clot gave an idea of the surface infection, and others from which serial paraffin sections were made permitted a study of the entire tissue. During cultivation, the flasks were rotated in a drum. The cultures were given fresh medium when the old medium was removed and cultured for tubercle bacilli on Loewenstein medium at periods of 1,2, 4, 6, 8, and 10 weeks, and 3, 4, and 6 months. The cultures were patched with chick plasma and extract at the 1 week period and 3 weeks period.

RESULTS

Fig. 1, 2, 3, 4, and Table I illustrate the growth curves obtained with the infected cultures. The highest concentration of bacilli, containing 45,000,000 infective units per flask (1 ml medium), caused depressed growth the first week of cultivation, and death of the cultures within a month. In the case of 10^{-6} dilutions of the original concentration, the cultures at first showed normal growth, then stimulated growth followed by depressed growth. Symbiosis was demonstrated by the surviving cells and bacilli at the end of 3 months. In these cultures free healthy cells continued to live in association with large masses of cords of tubercle bacilli.

At lower dilutions, between 10^{-2} and 10^{-6}, growth was stimulated during the first weeks, and depressed growth did not develop until after the first month of cultivation. During the third month of cultivation, cords of tubercle bacilli covered most of the tissue cultures, the cells having lysed into separate nests, usually making area studies impractical beyond this point. The fluid phase contained bacteria at all times, excepting the highest dilution. When the medium from these cultures (10^{-6}) was cultured for tubercle bacilli on Loewenstein medium, no tubercle bacilli could be demonstrated on the 7th day after infection. Subsequent cultures were positive.

Vacuolization and increased basophilia of the cytoplasm was seen in infected cultures, and a tendency to form multinucleated cells, some cells containing as many as 12–14 nuclei. Paraffin sections revealed that the tubercle bacilli were present in cord formation between cells, and that the cultures

contained many intracellular bacilli, lying in parallel strands. These ce
were often in excellent condition. The most luxuriant cords of tubercle baci
formed in the more dilute cultures. See Figs. 5 and 6.

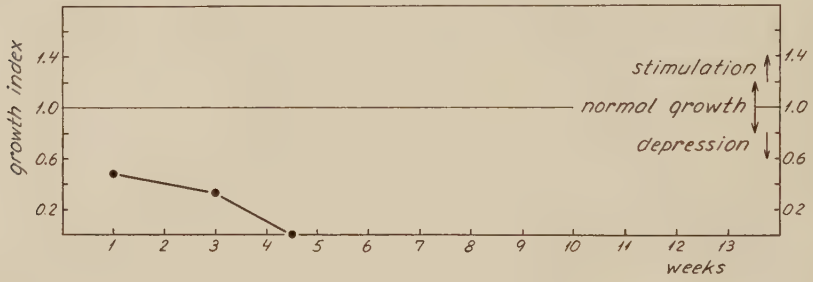


Fig. 1. Growth curves for tissue cultures with concentrated bacterial suspension (45,000,000/c

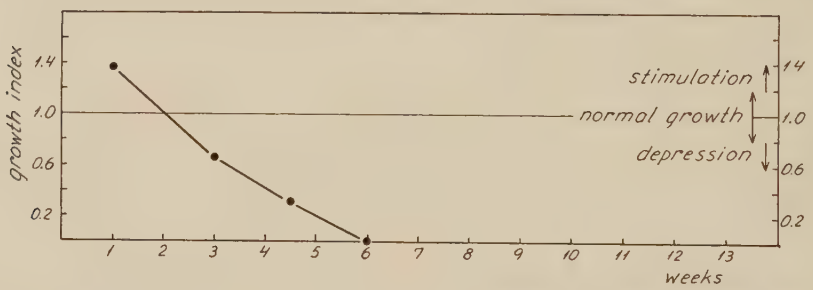


Fig. 2. Growth curves for tissue cultures with dilute bacterial suspension (4,500,000–450,000/

In contrast to the peripheral mode of infection sometimes seen with vi
agents in tissue culture, infection with the tubercle bacillus showed init
cell destruction and formation of bacillary cords at the center of the tiss
cultures, leaving a healthy ring of cells surrounding a destroyed area. U
published studies with polio infected cultures showed initially damag
peripheral cells and a more or less healthy core.

Large masses of tubercle bacilli in cord formation developed over t
central portions of the stimulated cultures, after first growing without co
formation in the lysed area formed by the tissue cultures in their thick cent
zones when cultured over a long period without transfer.

Masses of tubercle bacilli developed in areas where cells were grow
rather than on the clot itself, in contrast to the type of infections frequen
seen in contaminations with the staphylococcus and other bacteria. F
quently a film of tubercle bacilli covered the total surface in the flask, w
grossly detectable large masses of cords lying over the center of each cultu

Although growth of bacilli was not limited to the central area of the cultures, t was repeatedly observed that large masses of cords developed there. Special Manheimer–Seligman stains were done with the assistance of Dr. Virginia

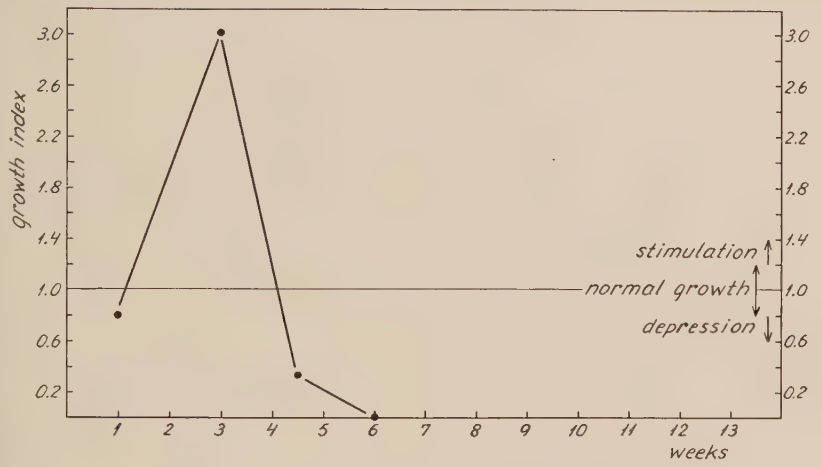


Fig. 3. Growth curves for tissue cultures with very dilute bacterial suspensions.

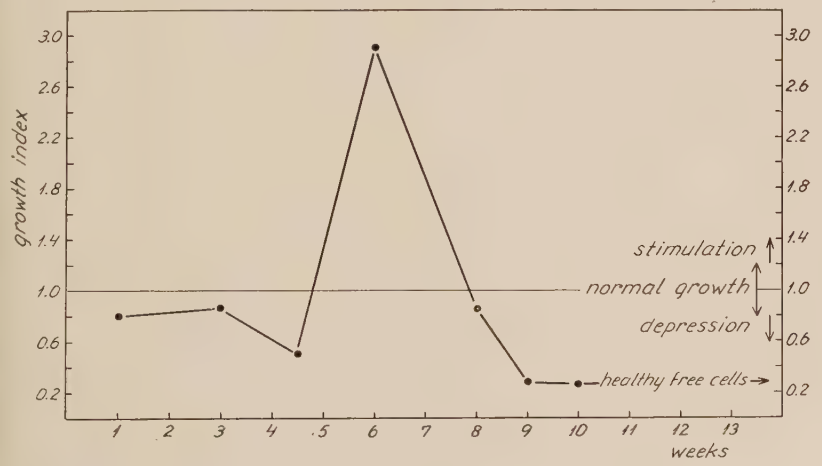


Fig. 4. Growth curves for tissue cultures with less than 100 bacteria/cc.

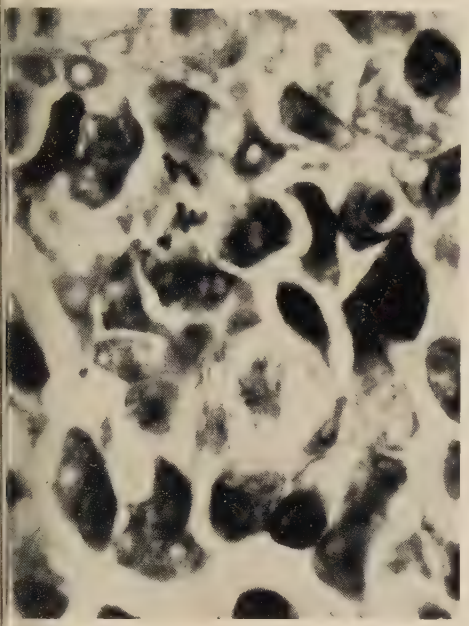
Green, and it was shown that these areas were usually phosphatase positive, n both controls and experiments. In old cultures a ring of calcium deposition ould be observed around the edge of the original central piece of the culture rom which the new cells grew. Astrup and Henrichsen [1] have shown that he ring is formed by calcium phosphate. Fischer, Fogh and Bohus Jensen

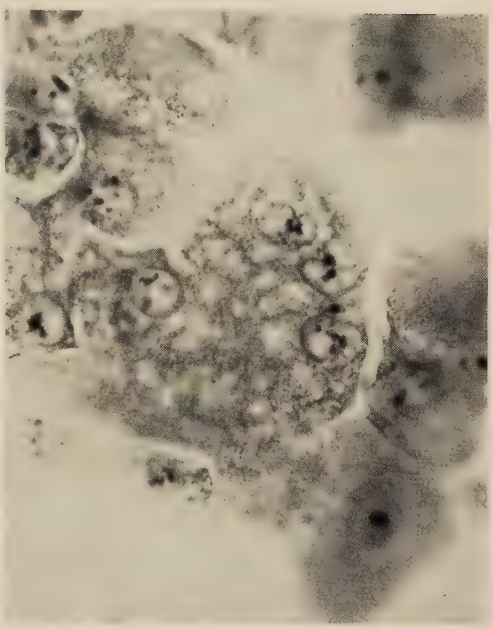
TABLE I

Tissue culture growth indexes.

No. Tubercle bacilli	1st week		3 weeks		1 month		6 weeks		8 weeks		2 months		10 weeks		3 months		6 months	
	T.B.	Index	T.B.	Index	T.B.	Index	T.B.	Index	T.B.	Index	T.B.	Index	T.B.	Index	T.B.	Index	T.B.	Index
Experiment I																		
Conc. susp.	+	0.48	+	0.34	+	0		d*		d		d		d		d		d
10^{-1}	+	0.92	+	0.29	+	0.10	+	0		d		d		d		d		d
10^{-2}	+	1.37	+	0.66	+	0.31	+	0		d		d		d		d		d
10^{-3}	+	0.80	+	3.16	+	0.33	+	0		d		d		d		d		d
10^{-4}	+	1.04	+	1.14	+	0.76	+	0.87	+	0		d		d		d		d
10^{-5}	+	0.80	+	0.86	+	0.50	+	2.91	+	0.85	+	0.28	+	0.27	+	healthy free cells	+	healthy free cells
10^{-6}	−	0.80	+	1.17	+	2.58	+	0.77	+	0.65	+	0.61	+	0.38	+	helthy free cells	+	healthy free cells
Experiment II																		
Con. susp.	+	0.48	+	0.20	+	0		d		d		d		d		d		d
10^{-2}	+	0.93	+	1.28	+	0		d		d		d		d		d		d
10^{-4}	+	1.63	+	1.44	+	6.38	+	1.83	+	2.72	+	2.67	+	0	+	d		d
10^{-6}	−	1.73	+	2.27	+	4.93	+	5.05	+	1.48	+	2.06	+	0.09	+	healthy free cells	+	healthy free cells

* dead.





g. 5 (*left*). Human epidermoid carcinoma tissue culture cells Af (H. Ep. No. 2) infected with rulent *Mycobacterium tuberculosis* var. *hominis*. Intracellular growth of bacilli in parallel ar- ngement can be seen with active tissue culture growth. × 1350.
g. 6 (*right*). Human epidermoid carcinoma tissue culture cells Af (H. Ep. No. 2) showing the pical giant cell formation found in cultures infected with virulent *Mycobacterium tuberculosis* ar. *hominis*. Multinucleation, large vacuoles, and small bead-like vacuoles can be seen.

)] in investigating the preservation of cells in the central part of tissue cultures, ave pointed out that the central part has the lowest oxygen tension and the west mitotic activity. Fell, Brieger, Smith *et al.* have pointed out in using xplants that there is frequently a central necrosis and a healthy ring of ells in normal and tuberculous infected tissue cultures.

In the experiments presented, the proportion of cancer cells to tubercle acilli was reversed in the dilute suspension cultures over a period of 6 nonths. The 0 day 10^{-6} cultures contained 45 infective units in the free ate, and several million tissue cells in solid sheet formation. At the end of ie half year period of cultivation, relatively few tissue cells were present, hiefly in the free state, and large solid masses of tubercle bacilli in cords ere present. The tubercle bacilli seemed to have grown at the expense of ie tissue cells; however, both bacilli and cells looked healthy at this stage. n the concentrated suspension cultures, the cells looked toxic and degener- ted in the presence of approximately the same number of bacilli after a week

or two. In one preliminary experiment, prolonged for 6 months, the cel began to show degeneration, and the clumps of tubercle bacilli became friab and stained unevenly. A small nest of cells was transferred, and after t cell growth increased, cords of healthy looking tubercle bacilli appear over the center of the culture. The cells in the controls and experiments aft a few months of semistarvation, were smaller, had fewer nucleoli and fin nuclear membranes. The cells, fewer in number in the experimental cultur at this stage, looked better than the cells in the control cultures.

DISCUSSION

Preliminary work and the studies presented suggest four types of reactio The tissue cultures receiving concentrated amounts of tubercle bacilli (Fig. showed initial depressed growth, approximately half the growth of the co trol, and relatively rapid degeneration. Cultures receiving fewer bacilli (Fi 2, 3) showed normal to stimulated growth with subsequent death. Tiss cultures receiving very dilute suspensions of bacilli, however, showed symbiotic prolonged survival with large grossly visible masses of bacte which developed in the culture (Fig. 4).

During a year of collaboration with Albert Fischer, the author investigat tissue cultures infected with air contaminants and with a variety of speci organisms. Unpublished results indicated that the bacterial infections studi frequently caused tissue culture stimulation [12]. Infected tissue cultu grew more luxuriously than uninfected tissue culture controls during t initial stages of infection, i.e., before the infection could be perceived by l power microscopic examination. After the infection became massive, d generation processes set in. Although it is difficult to separate the gene effect of infection from the specific effect of the tuberculous infection, w is in progress investigating the possibilities. Unpublished work suggests t there is a cytotoxic substance in filtered Dubos tween albumin culture tubercle bacilli, and that a polysaccharide fraction described by Sorkin a Boyden [22] and cord factor [3] effect the cell growth.

SUMMARY

A pattern of growth in human epidermoid carcinoma tissue cultu infected with virulent *Mycobacterium tuberculosis* var. *hominis*, was describ The bacteria and tissue cells grew together for a period of several mont

The effects ranged from toxic and depressed growth with heavy suspensi

f organisms to a stimulated growth of tissue cells with survival of cells and acilli.

Multinucleated cells appeared in infected cultures, and a fine vacuolization of the cytoplasm.

The author wishes to express appreciation for statistical evaluation from Statistician M. W. Bentzon and for the technical assistance of Rita Hansen.

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CONTRIBUTIONS TO THE QUANTITATIVE INTERPRETATION OF ELECTRON MICROSCOPE PICTURES

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During the last ten years exceptionally rapid advances, in the technic and preparative fields alike, have led to the development of histoanalytic electron microscopy into an effective means for carrying out biological r search. The main progress has so far been achieved in the morphologic sphere where the practical improvement in resolving power compared to lig microscopes has revealed a number of structures hitherto unknown. On th other hand, there has been no lack of attempts to use the electron microsco as an instrument for quantitative analysis. For example, attempts have be made to determine the atom-specific losses of energy in inelastic scatterin The differences in velocity of the electrons passing through, resulting fro this loss, can be measured by suitable means. Although elements with a atomic number of up to 26 are suitable for analysis, the information obtain is of limited value since the resolving power of the method is depende upon the focusing of the electron beam on the object to be examined [1 Trials to exploit the increased emission of a $BaCO_3$–$SiCO_3$ layer on t cathode by means of calcium and magnesium in the object are also hanc capped by insufficient resolving power [14].

Marton and Schiff [13] carried out calculations relating to image interpret tion for the electron microscope. Hall examined particles of tobacco mosa virus by means of a photographic process [18] and the absorption of me salts in a quantitative study. Krüger-Thiemer also used a photographic pr ess to determine the relative mass concentration of bacteria [10]. Howev these examinations only involved isolated objects.

As a result of the development of the ultra-thin section method into routine process (see [15, 16]), it is now possible to examine any kind of tiss with a layer thickness of about 200 Å. In this way it is possible to make u of the increased resolving power of the electron microscope and of structu analysis to arrive at chemical-physical statements regarding the object.

[1] Agfa Aktiengesellschaft für Photofabrikation, Leverkusen.

Considerable experience in ultraviolet microspectrophotometry, microradiography and interference microphotometry is available for cytochemical work of this nature (see Caspersson, [4]). These methods can be extended in a suitable manner by means of the improved resolving power of an electron microscope. In this paper it is intended to examine the possibilities of quantitative work with the electron microscope. In order to provide formulae which can be applied in practice, the basic principles will be treated first, to be followed by a discussion of the application, and the limits of application, of the results obtained.

General Solution of the Problem

The electron microscope picture of an object is the intensity distribution of a beam of electrons which is homogeneous with regard to direction and velocity after passing through the object. This intensity distribution is usually rendered visible by a fluorescent screen or by photographic effect. However, if one wishes to draw conclusions from the "picture" of an object produced in this manner, it is necessary to know the effects leading to modulation in intensity in the object. In spite of a number of excellent accounts [2, 7, 18] on the production of electron microscope pictures, it may be justified, with regard to our problems and because of the connection of ideas involved, to deal briefly with the alteration of the beam of electrons in the object. (The alterations of the object caused by the beam of electrons will not be considered for the moment, nor the loss of energy experienced by some of the electrons due to ionisation.)

Our questions are thus (*a*) which characteristics of the object point determine the brightness of the image point? (*b*) What conclusions can be drawn regarding the object from the contrast relationship existing in the picture? (Hereinafter, contrast K shall denote the natural logarithm of the relationship between two intensities.)

With the limitations imposed we need only observe the effect of scattering of the electrons in the object. When an electron comes within the sphere of action of an atomic nucleus it is diverted from its original direction. Let us describe such an event as impact. It is clear that the diversion will be greater, the deeper the electron penetrates into the nuclear field, the longer it stays there, i.e. the more slowly it travels and the more heavily the nucleus is charged. If a certain deviation in angle from the direction of incidence is exceeded, the electron does not enter the effective aperture of the electron optical system and no image is formed. Thus the variations in intensity are the image of the object areas with different scattering power.

In order to arrive at quantitative data we must know the angular distribu tion of the electrons after passing through a layer (regarded as homogeneou for the purpose of our deliberations). Only then can we calculate the propor tion falling into the aperture.

Some of the electrons striking the layer at right angles will undergo deviating impact when passing through it. The probability of finding a electron deviated by the angle ϑ from its original direction after one impac is expressed as follows:

$$f_1(\vartheta)\,\vartheta\,d\vartheta,$$

where $\vartheta\,d\vartheta$ is the solid angle; azimuth is suppressed owing to cylindrica symmetry, sin $\vartheta \approx \vartheta$ applied.

However, some of the electrons are diverted twice, or generally n times, s that

$$f_n(\vartheta)\,\vartheta\,d\vartheta$$

represents the probability of deviation by ϑ after n impacts.

If, in addition, we know the probability W_n that n impacts occur, th resulting angular distribution $f(\vartheta)$ behind the object is simply expresse according to the laws of probability calculus as

$$f(\vartheta)\,\vartheta\,d\vartheta = \vartheta\,d\vartheta \sum_0^\infty W_n f_n(\vartheta) \qquad (3$$

or, if I_0 is the incident intensity, the result will be:

$$i(\vartheta)\,\vartheta\,d\vartheta = I_0\,\vartheta\,d\vartheta \sum_0^\infty W_n f_n(\vartheta). \qquad (4$$

For the formation of an image, however, only those electrons are detecte whose deviation is less than the aperture angle θ, so that, in the image plan the intensity

$$I = I_0 \int_0^\theta \vartheta\,d\vartheta \sum_0^\infty W_n f_n(\vartheta) \qquad (5$$

is effective.

Specialisation of the Formula for Practical Work

The impact number probability W_n.—According to Poisson the probabil W_n is shown as

$$W_n = e^{-m}\frac{m^n}{n!}. \qquad (6$$

m stands for the mean impact number in the layer, whilst n, as alrea

ıentioned, represents the actual number. The function W_n has a maximum : $n = m$ which is more pronounced the smaller m is, so that m determines om which n the quantities W_n have become negligible in the sum (3). The relationship of the maximum probability W_m to the W_{m+1}, which is reater for one impact number, is $(m+1)/m$).

In addition, W_n as a genuine probability fulfils the condition

$$\sum_{0}^{\infty} W_n = 1.$$

The mean impact number m.—The mean number of impacts experienced y an electron passing through the object is a distinct and essential quantity or describing the scattering process.

This impact number m is mainly dependent upon the thickness and density the object but less so upon its chemical composition. The theory [11] of astic scattering gives the following formula for m:

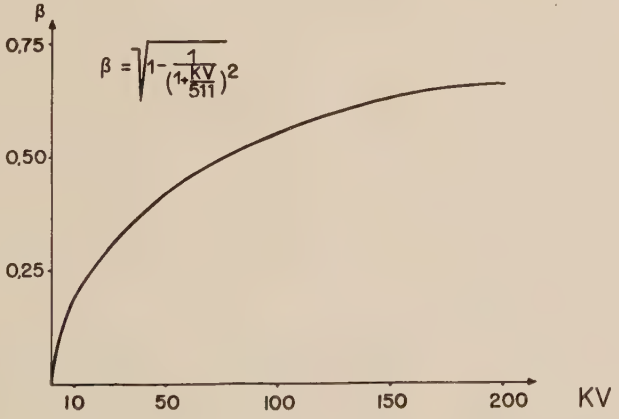
$$m = 0.88 \frac{Z^{4/3}}{A} \cdot \frac{10^4}{\beta^2} t, \tag{5}$$

ı which A is the atomic weight,

Z is the atomic number,

$\beta = v/c$ is the ratio of the velocity of the electron to the speed of light (in the case of our special examination $\beta = 0.417$ for an accelerating voltage of 50 kv in the electron microscope), (see Fig. 1), and

t is the mass thickness of the object in g/cm². (The relationship to the thickness is $d = t/\varrho$; d in cm; ϱ = density of the object in g/cm³.)



ig. 1. β = ratio of the velocity the electrons to the velocity light as a function of the acelerating voltage.

Where the layer thickness is given, the mean impact number in variou substances is mainly dependent upon their density. As shown in Fig. 2, th quantity $x = Z^{4/3}/A$ causes only a slight change for the whole periodic systen whilst the densities of solid substances vary by the factor 1:20.

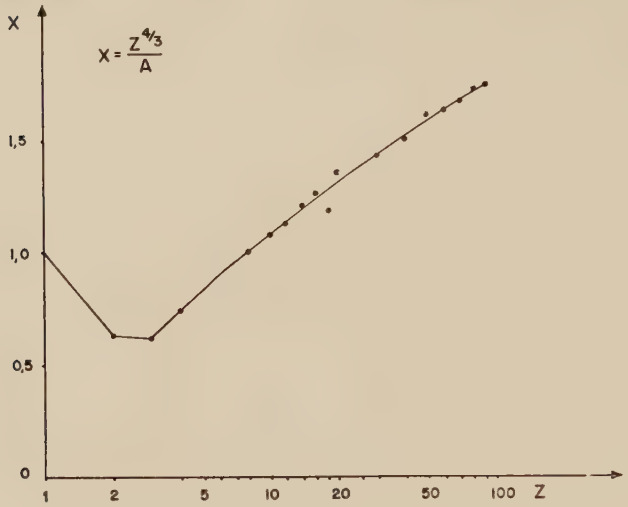


Fig. 2. $x = Z^{4/3}/A$ as a funct of the atomic number Z.

The mean impact number is simple in its connection with the effecti cross-section. If σ represents the effective cross-section of an atom determini the scattering of electrons, then 1 cm² of the penetrated object having t thickness d is covered with the area m effective for the scattering

$$m = \sigma \cdot N \cdot d$$

if N represents the number of atoms per cm³ scattering centres. The me number of impacts is thus revealed as the ratio

$$\frac{\text{Effective surface}}{\text{Total surface}} = m.$$

If (5) and (6) are compared and it is considered that

$$N = \varrho \frac{L}{A} \qquad (L = \text{Avogadro number}),$$

this results in

$$\sigma = \frac{m A}{t L} = \frac{0.88}{6.02} \cdot \frac{Z^{4/3}}{\beta^2} \cdot 10^{-19}\,(\text{cm}^2)$$

ecoming $\sigma = 0.840 \cdot Z^{4/3} \cdot 10^{-19}$ (cm²)
ecially for 50 kv.

If, as is usually the case, the substances to be examined are not chemically omogeneous, then it follows from what has been said above that the mean npact number of the compound is taken as the sum of the mean impact umber of the individual components.

Thus, if the compound is designated by the weight ratios of the kinds of oms concerned, this means that

TABLE I*

$m = 5.06 \cdot 10^4 \cdot X \cdot \varrho \cdot d$ (for 50 kv).

Compound	$C_i X_i = X$	$X \cdot \varrho$	$d = 200$ Å	$d = 300$ Å
Animal proteins	0.949	1.2622	0.1278	0.1917
Fibrin	0.948	1.2608	0.1276	0.1914
Elastin	0.945	1.2569	0.1272	0.1908
Collagen	0.948	1.2608	0.1276	0.1914
Amyloid	0.960	1.2768	0.1276	0.1938
Thymus-histone	0.946	1.2582	0.1274	0.1911
Plant proteins	0.946	1.2582	0.1274	0.1911
Desoxyribonucleic acid	0.977	1.4655	0.1484	0.2226
Ribonucleic acid	0.980	1.4700	0.1488	0.2232
Glycogen	0.960	—	—	—
Tripalmitin	0.931	0.8060	0.0816	0.1224
Tristearin	0.930	0.8018	0.0812	0.1218
Triolein	0.930	0.8510	0.0862	0.1293
Trilinolin	0.929	—	—	—
Cerebrosides	0.942	—	—	—
Haematin	0.971	—	—	—
Parlodion	0.969	1.609	0.1628	0.2442
Zapon varnish	0.955	—	—	—
Cellulose dinitrate	0.965	1.602	0.1622	0.2433
Cellulose trinitrate	0.967	1.605	0.1624	0.2436
Cellulose tetranitrate	0.969	1.609	0.1628	0.2442
Cellulose pentanitrate	0.971	1.612	0.1632	0.2448
Cellulose hexanitrate	0.972	1.614	0.1634	0.2451
Methylmethacrylate	0.949	1.084	0.1098	0.1642
Butylmethacrylate	0.940	1.128	0.1142	0.1713
Bensoylperoxide	0.937	—	—	—

* Chemical analyses partly taken from Lindström [12].

$$m = \sum m_i = d \sum \cdot N_i \cdot \sigma_i$$

$$= 0.88 \cdot \frac{10^4}{\beta^2} \cdot t \sum C_i \cdot \frac{Z_i^{4/3}}{A_i} \qquad (7$$

$$= 0.88 \cdot \frac{10^4}{\beta^2} \cdot t \left(\frac{Z^{4/3}}{A}\right) \text{ eff.}$$

For 50 kv in particular

$$m = 5.06 \cdot 10^4 \cdot t \cdot \left(\frac{Z^{4/3}}{A}\right) \text{ eff.}$$

The sum $\sum C_i \; Z_i^{4/3}/A_i$ gives us the definition of the effective value $Z^{4/3}/A$ the corresponding compound. The effective values for the most importa biological compounds are listed in Table I.

Fig. 3 gives an idea of the magnitude of impact numbers encountered practice; m is plotted as a function of the atomic number Z for various obje thicknesses (mass thicknesses in g/cm²). In the case of the density of t object $\varrho = 1$, which usually occurs with the small Z values of biologic objects in electron microscopy, the thickness numbers noted correspo to the particulars in Ångström units. In Fig. 4 the density of the elemer has been taken into account. The thickness of 500 Å in the section for ex mination, which can be obtained very easily by means of the section tec

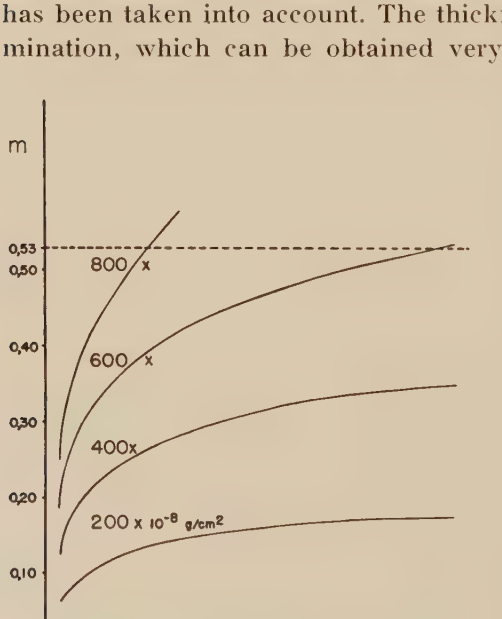
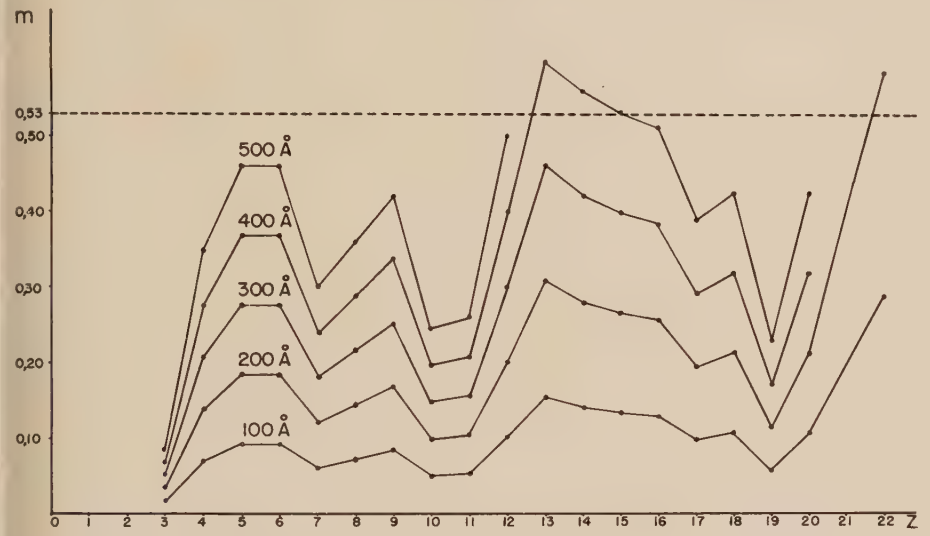


Fig. 3. The mean impact number m as a f tion of Z for different object thicknesse g/cm²).

ique developed nowadays, results in mean impact numbers in the region f 0.5 for pure elements to a Z figure of 20.

Validity range.—In general the infinite summation necessary for practical pplicability of formula (3) is not possible, and we are forced to discontinue ıe series with a final n and estimate the error. In the case of a given maximum



ig. 4 *a*. The mean impact number m as a function of Z calculated for distinct layer thicknesses Å (lower atomic numbers).

, the magnitude of the error is dependent upon the mean impact number hich is the factor determining the convergence. Since $f_n(\vartheta) \leq 1$ always holds ood, the majorant of the sum in (3) is $\sum_0^\infty W_n$ the convergence criterion $V_{n+1}/W_n = m/(n+1)$.

This is understandable since, as already mentioned, the most frequent npact number n is equal to the mean impact number m. If, for example, $= 0.4$, the singly scattered electrons will amount to only 40 per cent, namely $V_1/W_0 = 0.4 = m$, of the unscattered, whilst those scattered twice will total nly 8 per cent; generally speaking: If $m \ll 1$, only single scattering is ecisive.

Thus the sum in (3) consists of two terms only, namely the unscattered roportion ($n = 0$) and the proportion scattered once ($n = 1$). If we impose ıe stipulation that the proportion scattered more than once should not xceed 10 per cent, we arrive at the figure $m = 0.53$. The figure 0.53 is the

solution to the transcendental equation $\sum_{2}^{\infty} W_n = 0.1$. (The error is even smalle since a large proportion of the electrons scattered several times contribu to form the image.)

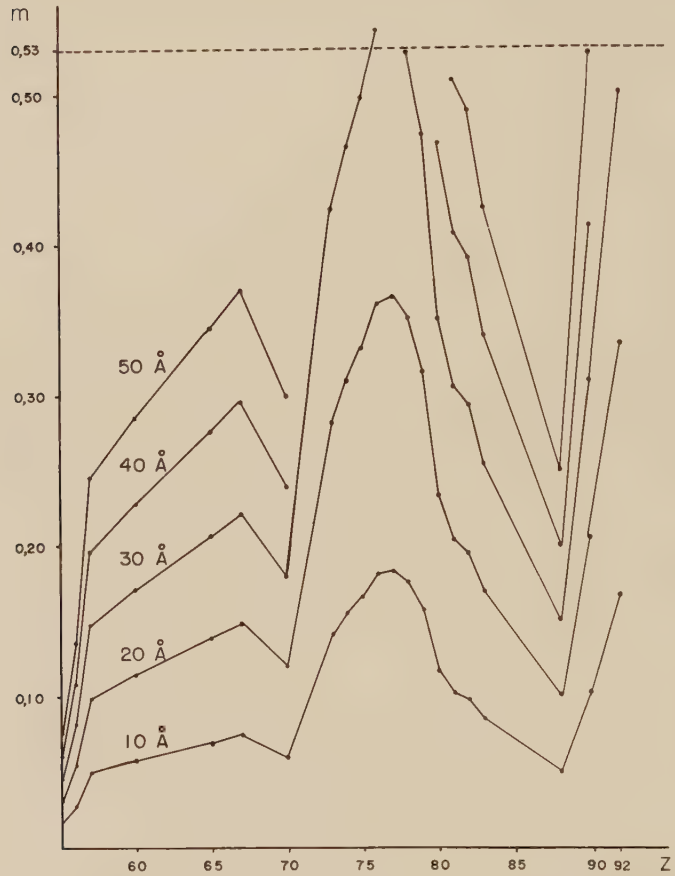
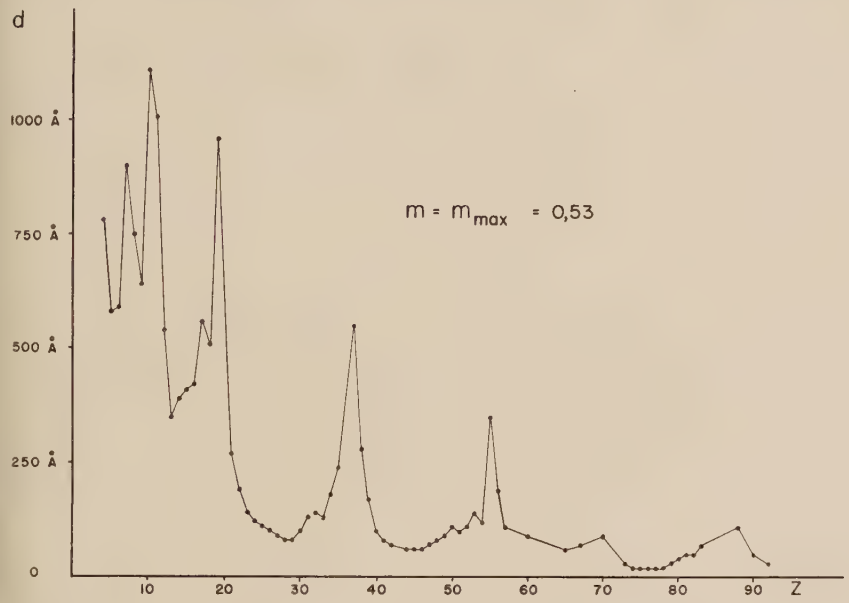


Fig. 4 *b*. As Fig. 4 c higher atomic numb

According to formula (5), this limitation of the impact number means practice, a fixing of the maximum object thickness t, for all the quant occurring in (5) are not freely selective but are determined either by appar or by the object itself. In Fig. 5 the maximum permissible object thickne of pure (!) elements for our validity range are represented in Ångström u The "periodicity" is caused by the variation in the volumina of the at

Standardised angular distributions.—In accordance with the fixed lin tion we need only consider the values $f_0(\vartheta)$ and $f_1(\vartheta)$. $f_0(\vartheta)$ is the distr

on of the unscattered electrons and thus of those which fall on the object. 'e shall presuppose these to be parallel beams, i.e. only electrons are in- olved which have the angle zero (determined vertically to the object lane). The probability to find electrons within angles differing from zero zero. (We thus disregard the initial aperture of the beam.) A distribution



g. 5. Maximal section thickness as a function of Z in the given validity range ($m = 0.53$). For planation see text.

this kind is described by the delta function, the equation being expressed follows:

$$f_0(\vartheta)\,\vartheta\,d\vartheta = \delta(0)\,\vartheta\,d\vartheta. \tag{8}$$

he distribution f_1 of the electrons scattered once (Fig. 6) is known for the reened coulomb field of the atomic nuclei and results in the equation

$$f_1(\vartheta)\,\vartheta\,d\vartheta = 2\vartheta^2(\vartheta^2 + \vartheta_0^2)^{-2} \cdot \vartheta\,d\vartheta \tag{9}$$

which the quantity ϑ_0 is the so-called screening angle $Z^{1/3}\{(1-\beta^2)/\beta\}$, in grees. This renders the distribution dependent upon Z of the object (Fig. 7). *Evaluation of formula (3).*—If we consider the special intermediate results the preceding paragraphs in formula (3), this can be set out as follows:

$$I = I_0 e^{-m} \int_0^{\theta} \vartheta \, d\vartheta \, (f_0 + m f_1)$$

$$= I_0 e^{-m} \left\{ 1 + m \int_0^{\theta} f_1(\vartheta) \, \vartheta \, d\vartheta \right\} . \qquad (3$$

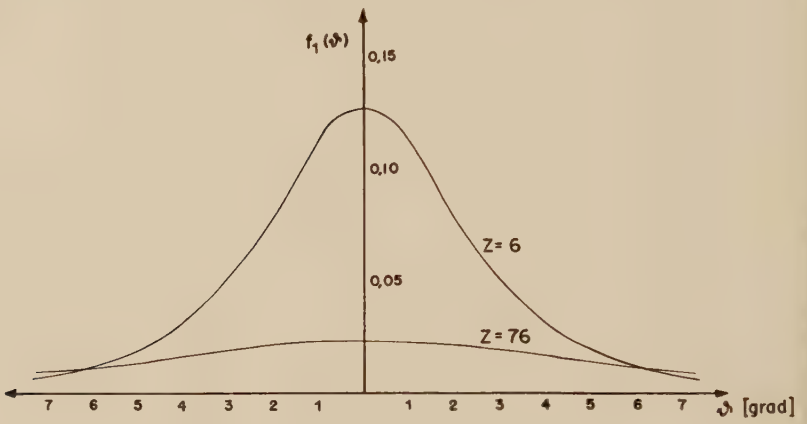


Fig. 6. Angular distribution $f_1(\vartheta)$ of single scattered electrons as s function of the angle $Z = 6$ and $Z = 76$.

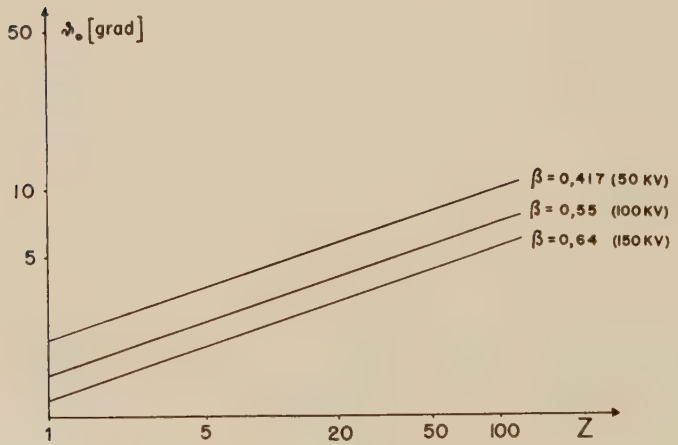


Fig. 7. Screening angle a function of Z at diff acceleration voltages (β

Due to the special form (9) for $f_1(\vartheta)$, the integral can easily be evaluate

$$\int_0^{\theta} f_1(\vartheta) \, \vartheta \, d\vartheta = \frac{\theta^2}{\vartheta_0^2 + \theta_2} .$$

f we designate this term as k for short, the following result is obtained:

$$I = I_0 e^{-m}(1 + mk). \tag{3b}$$

since mk is small against one (θ is in the magnitude of 10^{-2} degrees), we an re-write (3b) as:

$$I = I_0 e^{-m(1-k)}. \tag{10}$$

Hall [9] also obtains a formula of the same type by another method. The onstant k is introduced only phenomenally; multiple scattering is also lisregarded, even if implicitly.

In our determinations k is revealed as an apparatus constant which shows slight increase with the atomic number of the object as a result of the screening angle which still occurs.

Object Models

In this section it is intended to apply the formulae obtained above for the study of contrast relationships of electron microscope pictures. We shall commence with simple object models in order to demonstrate the data of the object which must be known to be able to arrive at quantitative information.

(*a*) If we place an object of uniform composition and density in an electron microscope beam having the intensity I_0, we shall encounter only the intensity I in the image plane. At the edge of the object we measure the contrast (Fig. 8)

$$K = \ln \frac{I_0}{I}.$$

Our formula gives for

$$K = m(1-k). \tag{11}$$

Thus we see that, as far as the electron microscope is concerned, only the mean impact number m plays a part in determining the contrast. However, according to formula (7) the various objects can have the same m, provided the product of $t \sum C_i x_i$ is equal. This is the reason for a certain degree of ambiguity of electron-microscope picture evaluation. In other words, the thickness, density and x can vary considerably without exercising any effect upon the mean impact number, so long as the product in question remains the same. As far as the usual biological objects examined in an electron microscope are concerned, certain conditions are given. Thus, generally speaking, the embedding material, nowadays consisting of mixtures of methyl methacrylate and butyl methacrylate, can be regarded as constant in composition.

From Table I it can be seen that x and m are of the same order of magnitud for the object components of interest in this case. In addition, Table I reveal that x can be regarded as equal, for example, for animal and vegetable pro teins. Since the thickness of the object being examined is fixed by an ade quate section technique, only alterations in the density of the pure biologica object are effective. If, on the other hand, density and chemical compositio

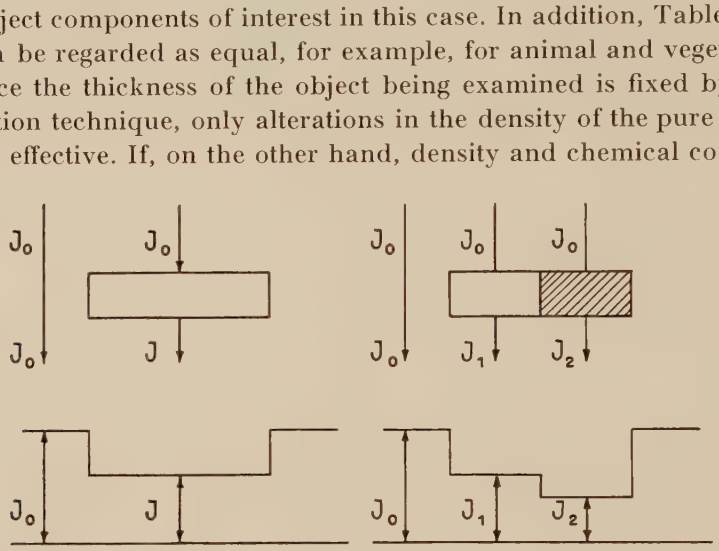


Fig. 8 (*left*) and Fig. 9 (*right*). Simple object models and resulting distribution of beam intensit

of the microscopic layer, e.g. of the embedding material, is known, formu (10) in Hall's [8] process can be used to provide details of its thickness. It necessary of course, as already mentioned by Hall the take account of t contrast-diminishing scattered radiation lying over the whole picture. what follows, I will denote, in each case, the already corrected values f which the derived formulae are valid.

(*b*) The object consists of two areas of different mean impact numbe but of equal thickness. Beyond one area we find the intensity

$$I_1 = I_0 e^{-m_1(1-k_1)}$$

and

$$I_2 = I_0 e^{-m_2(1-k_2)} \qquad \text{(Fig. 9)}.$$

beyond the other.

This time the contrast we measure is

$$\ln \frac{I_2}{I_1} = m_1(1-k_1) - m_2(1-k_2).$$

Since k varies only slightly with the atomic number and, in addition, alwa remains small in comparison to 1, we obtain

TABLE II

Z	Element	$X=\frac{Z^{4/3}}{A}$	ϱ	$X \cdot \varrho$
1	H	1.000	0.09	0.09
3	Li	0.623	0.53	0.33
4	Be	0.704	1.85.	1.37
5	B	0.790	2.3	1.82
6	C	0.909	2.0	1.82
7	N	0.956	1.25	1.19
8	O	1.000	1.43	1.43
9	F	0.985	1.69	1.66
10	Ne	1.076	0.9	0.96
11	Na	1.064	0.97	1.03
12	Mg	1.129	1.74	1.97
13	Al	1.133	2.7	3.05
14	Si	1.202	2.3	2.76
15	P	1.193	2.2	2.62
16	S	1.258	2.0	2.52
17	Cl	1.233	1.55	1.91
18	Ar	1.181	1.78	2.10
19	K	1.296	0.86	1.12
20	Ca	1.354	1.55	2.09
76	Os	1.68	22.5	37.80
78	Pt	1.71	21.5	36.77
79	Au	1.72	19.3	33.20
80	Hg	1.72	13.5	23.22

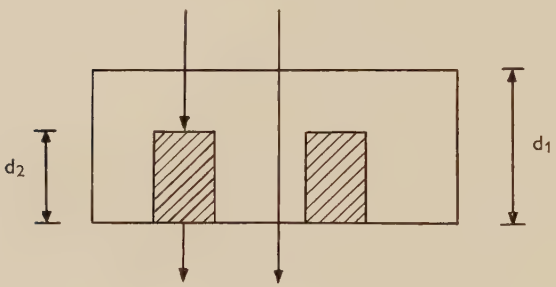
$$K = m_1 - m_2 \qquad (12)$$

The contrast turns out to be the difference in the mean impact number.

If we know the chemical composition of both substances and the density of only one, we can insert this latter as a reference system in regard to the object density and arrive at the unknown density of the other. If we also determine the surface of the object by planimetering and using the familiar enlarging scale, this investigation is equal to "weighing by an electron microscope".

(*c*) Above, under (*a*) and (*b*), we have dealt with the problem of contrast. It is more difficult to judge the possibility of conclusions regarding the object from the picture. We have previously seen that it is always necessary to know two of the three quantities of interest—density, thickness, or effective

$Z^{4/3}/A$, to be more precise, their local distribution—in order to determine the third unknown quantity. Reference has already been made to the impossibility of a chemical analysis due to the ambiguity of $(Z^{4/3}/A)_{\text{eff}}$ which was mentioned (12). In spite of this there is still a large range of application particularly with the assistance of suitable reference systems. Since an unambiguous conclusion from the picture regarding the object requires, from the viewpoint of electron microscopy, too many data on the object, our considerations will be based on an object model as nearly as possible approaching reality and where the deviations in density or X deviations can still be detected by measurement.



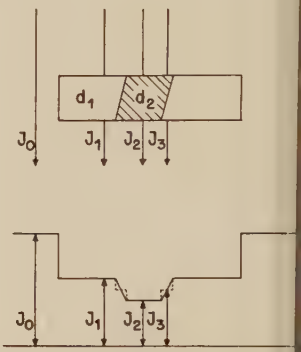


Fig. 10 (*left*) and Fig. 11 (*right*). Object model corresponding to a frequently occurring object typ

First a formula for the contrast has to be determined, if the density in the object as well as the chemical composition varies.

In a substance 1, (d_1, ϱ_1, x_1), a substance 2, characterised by the corresponding values, is embedded. The weight ratio of the solid matter of both substances is expressed as

$$q = \frac{\text{weight 1}}{\text{weight 2}}.$$

The first model to be presented is that shown in Fig. 10. The embedded substance is distributed in places with a mean thickness d_2, the covered fraction of the total surface in the electron microscope picture being α (Figs. 10 and 11).

The maximum contrast, such as results with the path of the beam shown in Fig. 11, is also calculated in this case from the impact number difference. According to (5)

$$\left.\begin{aligned} m &= 0.88 \cdot \frac{10^4}{\beta^2} x_1 \varrho_1 \cdot d_1, \\ m' &= 0.88 \cdot \frac{10^4}{\beta^2} x_1 \varrho_1 (d_1 - d_2) + x_2 \varrho_2 d_2, \\ K &= \frac{0.88 \cdot 10^4}{\beta^2} d_2 (x_2 \varrho_2 - x_1 \varrho_1); \end{aligned}\right\} \qquad (13)$$

For 50 kv in particular,

$$5.06 \cdot 10^4 d_2 (x_2 \varrho_2 - x_1 \varrho_1).$$

The mean layer thickness d_2 of the embedded substance is obtained simply from the following formulae:

$$\alpha \cdot d_2 (\varrho_2 - \varrho_1) + d_1 \varrho_1 = d_1 \cdot \varrho_{\text{total}},$$

$$\alpha \cdot d_2 \varrho_2 = q \cdot d_1 \cdot \varrho_{\text{total}}.$$

From this results d_2 at

$$d_2 = d_1 \cdot \frac{q}{\alpha} \cdot \frac{\varrho_1}{q \cdot \varrho_1 + (1-q)\varrho_2}$$

$$\frac{d_2}{d_1} = \frac{q}{\alpha} \cdot \frac{\varrho_1}{q \cdot \varrho_1 + (1-q)\varrho_2}$$

so that we obtain

$$K = 5.06 \cdot 10^4 \frac{q}{\alpha} \cdot \frac{\varrho_1 d_1}{q \cdot \varrho_1 + (1-q)\varrho_2} \cdot (x_2 \varrho_2 - x_1 \varrho_1). \qquad (13\,a)$$

If the object has a distribution of the embedded substance corresponding more to that represented in Fig. 12, the maximum contrast is reduced. The q value is found to be too small, since the homogeneous layer forms no contrast, and thus results, with the correctly determined q value, in high valued d_2, which can even exceed the known quantity d_1. In this way one at least has a check. In practice both distributions will be present in an object (Figs. 10, 11, 12).

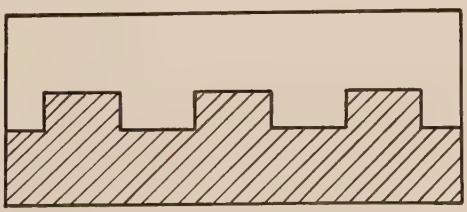


Fig. 12. See text.

Measuring the Contrast

The most precise method of measuring the distribution of intensity in the image plane is to use a Faraday cage. An apparatus of this type has been constructed for the RCA EMU type electron microscope for scanning and registering measurements in the image plane and for dealing with matter discussed here [1]. After substracting the background scattering the contrast is obtained as the logarithm of the intensity ratio. The disadvantages of such a procedure are the lack of a document, the point-by-point observation particularly the lengthy and harmful exposure of the object to the beam during measurement, and a lower resolution.

The photographic plate offers the second possibility for determining the contrast by photometric evaluation of the electron microscope picture and determination of the intensity relationship or contrast by means of a density curve characteristic for the photographic material used. The photographic process should be standardized appropriately to fulfil the requirements for quantitative work [5]. It is recommended on each occasion to run through the electron microscope pictures of the objects with an exposed density wedge the characteristic curve of which is compared with the density curve originally obtained. In this case the expenditure for apparatus is limited to an exact exposure arrangement which is part of the equipment on the latest electron microscopes.

As v. Borries [3] has shown, the characteristic curves of the Agfa plate can be represented with very great accuracy as

$$S = \gamma \log I + \beta. \qquad (1)$$

If we wish to determine the contrast $\ln I$, the following result is obtained from (14):

$$K = \Delta \ln I = \frac{\Delta S}{\gamma \cdot 0.43}. \qquad (1)$$

We have thus obtained a very simple result, namely, that the mean difference in impact number in the object is proportional to the difference in density on the image. $\gamma = \Delta S/(\Delta \log I)$ is the gradation of the photographic material (0.43 is the modulus of the logarithm).

Due to the low expenditure on apparatus involved, the photographic process has great advantages for those using the electron microscope in the biological field. If, however, routine examinations on inhomogeneous objects are to be carried out on a large scale, the experience of those engaged in ultraviolet and X-ray spectrophotometry of biological material has shown that it is barely possible to avoid the use of automatic processes [4].

Minimum Contrast in the Photographic Process

For example, with the aid of the γ value of 2.5 given by v. Borries [3] or the Agfa diapositive plate we can supply the lowest, still perceptible difference in impact number, since $S = 0.02$ represents the limit both for apparatus and from the physiological viewpoint.

$$K_{\min} = \frac{0.02}{\gamma \cdot 0.43} = \frac{0.05}{\gamma} = 0.02.$$

One should expect 0.05 as a difference in density which can still be evaluated well, so that the differences in impact number are in the order of $0.1/\gamma$.

As an example, let us use formula (13) to determine the layer thickness of osmium (Os), which just produces the minimum contrast when it occurs in an average biological material ($\varrho_1 = \varrho_{\text{animal protein}} = 1.33$ g/cm³). This figure is given by Cohn and Edsall [6] and is in general use today in interference microscopy:

$$x_1 \varrho_1 = 1.26 \qquad \text{(see Table I)}$$

$$x_2 \varrho_2 = 37.8 \qquad \text{(see Table II)}$$

$$d_2 \min = \frac{K_{\min}}{5.06 \cdot 10^4 (x_2 \varrho_2 - x_1 \varrho_1)} = \frac{5 \cdot 10^{-6}}{5.06 \cdot 36.54} = 2.7 \text{ Å}.$$

Thus the minimum determinable layer thickness of osmium is, for the above-mentioned initial values, in the region of 2.7 Å, while theoretically 27 Å is the maximum thickness of osmium in view of the limitation of impact number to 0.53; this provides sufficient scope.

In this example it is intended to calculate the deviations in density determinable on the electron microscope picture when applying the following values which frequently occur in practice.

Minimum contrast $K_{\min} = 0.04 \quad (\gamma = 2.5)$

$$d_2 = 200 \text{ Å}$$

$$X_1 = \left(\frac{Z^{3/4}}{A}\right) \text{carbon} = 1.129$$

$$\varrho_1 = 1 \text{ g/cm}^3$$

$$K_{\min} = 5.06 \cdot 10^4 \cdot d_2 X_1 \varrho_1 \left(\frac{\varrho_2 - \varrho_1}{\varrho_1}\right)$$

$$= \frac{\varrho_2 - \varrho_1}{\varrho_1} = \frac{0.04}{5.06 \cdot 2 \cdot 10^{-2} \cdot 1.129} = 0.36.$$

A 36 per cent deviation in density is therefore the visible minimum. If th layer thickness of the concentrated, embedded material is lower than tha shown in our example, the deviation in density must be greater to the sam extent and to an equal degree of perception. This conclusion is particularl significant for biological material which has been embedded without previou treatment with metal salts and is examined as a thin section, about whic more will be said below.

Application of the Results to Staining of the Objects with Metal Salts

Shortly after the first trials with the electron microscope in the biologic field it was seen that treatment with metal compounds of the object to b examined in a manner similar to the techniques in light microscopy produce considerable improvements in contrast and showed up a number of structur for the first time. The most suitable is osmium (in the form of osmiu tetroxide), which gives the greatest effect due to its maximum density of a the elements and also ensures, in its capacity as a fixing agent, unsurpasse maintenance of structure.

For reasons already mentioned, we specialise the formula (13) for prepar tions treated with osmium, i.e. the appropriate osmium values should b substituted for the quantities indicated with the suffix 2. The acceleratio voltage is fixed at 50 kv.

$$\frac{K}{d_1} = 5.06 \cdot 10^4 \frac{d_2}{d_1}(39.2 - x_1 \varrho_1), \tag{1}$$

$$\frac{d_2}{d_1} = \frac{q}{q + (1-q)\frac{23.5}{\varrho_1}} = 1/\alpha \cdot \tag{1}$$

Thus, in order to calculate the contrast, d_2 and $x_1 \varrho_1$ must be know however, to arrive at d_2 it is necessary to know d_1, and α. Since the x_1 value, biological objects, is always of the order of 1, but does not exceed its effect upon the contrast compared with $x_2 \varrho_2 = 39.2$ should be disregarde For ϱ_1 we substitute the already substantiated value of $\varrho_1 = 1.33$.

Diagram 13 provides an informative picture of the effect of the individu quantities.

In its first quadrant the ratio d_2/d_1 is entered as the function of the degr of surface coverage, and the dry weight relationship q as a parameter (1 It can be seen how, with the same α, d_2/d_1 rises with an increasing q where q remains constant, the layer thickness d_2 increases, if α decreas

The ordinates d_2/d_1 of the first quadrant are regarded in the seco quadrant as an abscissa of the graphical representation K/d_1 against $d_2/$

ı accordance with formula (16) the parameter is $x_1 \varrho_1$; as already mentioned, s effect is slight. Finally, the actual contrast is entered in the third quadrant.

If the contrast K is known from the electron microscope picture, and the nickness d_1 of the object by means of the reference system (in most cases the bject-free methacrylate of the section), the diagram provides information

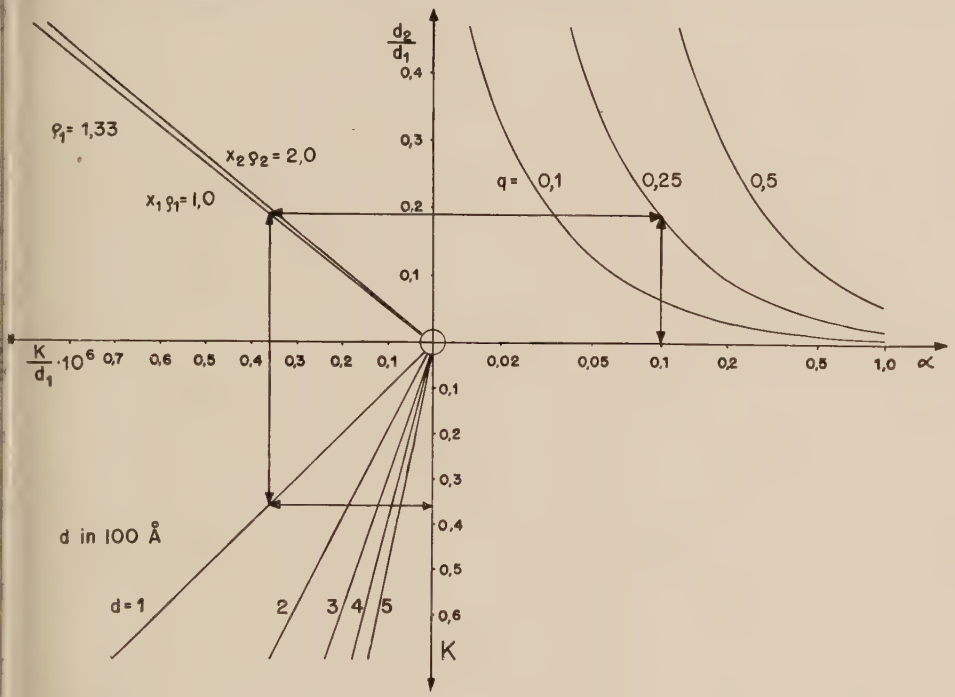


Fig. 13. Nomogram, see text.

garding the value d_2. The value K is located on the diagram, then crosses om here horizontally in the third quadrant to d_1, which has been deter-ined, proceeds vertically into the second quadrant to the corresponding $x_1 \varrho_1$ ncritical with biological objects); then the relationship d_2/d_1 can be read off the right in a horizontal direction.

If, in addition, the dry weight ratio q is known, it is possible to determine ıe relevant degree of coverage α or vice versa.

In this way we obtain a simple means of examining the agreement of our bject model with real objects, by comparing the q values, which are analytic-ly simple and certain to obtain, with the q values we have obtained with ıe aid of the diagram from the contrast and α values determined in the ectron microscope picture.

Application of the Results to Differences in Density in the Biological Object

In the evaluation of electron microscope pictures the question is often raise as to whether the contrast determined in an image area is caused by stainin i.e. absorption of salts of heavy metals form the accompanying object are

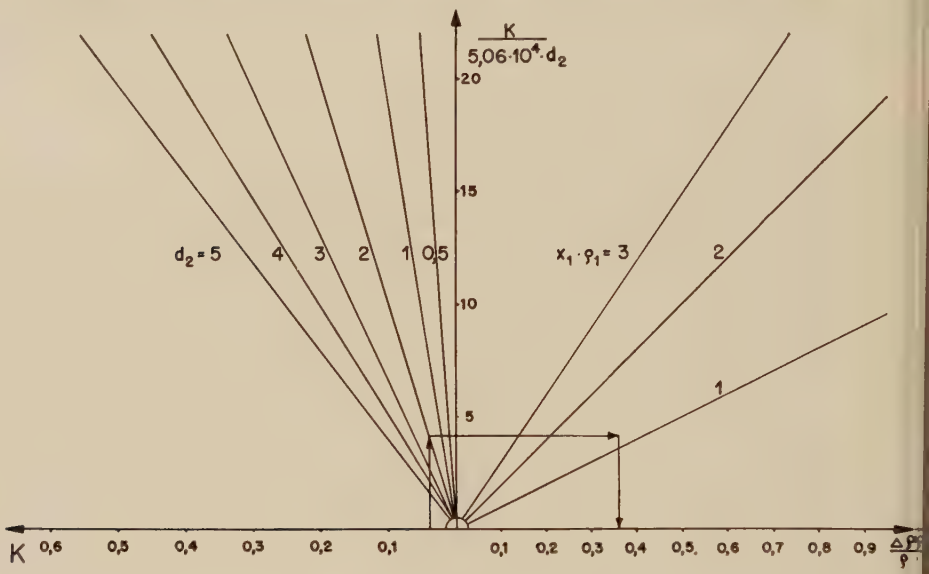


Fig. 14. Nomogram, see text.

or merely by its higher density in comparison to the surrounding area (in t case of the same material). In order to facilitate an answer to this question, us specialise the formula (13) and likewise represent it as a nomogra (again, 50 kv).

$$K = 5.06 \cdot 10^4 d_2 x_1 \varrho_1 \frac{\Delta \varrho}{\varrho}. \qquad (13$$

Since only elements having an atomic number of less than 20 are of biologi interest for such preparations, we can limit the $x_1 \varrho_1$ values up to 3. T mean layer thickness d_2 of the object area where the density is increase accessible to precise measurements if a reference system is incorporated the preparation and sectioned together with the object. For this purp pure elements of the lower atomic numbers are particularly suitable. C proceeds, in a manner similar to Fig. 13, from the calculated contrast, wh is entered as the ordinate of the second quadrant, vertically and upwa to the thickness d_2, and from there horizontally into the first quadrant up

e corresponding $x_1 \varrho_1$ value, whereupon the pertinent $\Delta\varrho/\varrho$ value is found
ı the abscissa of the first quadrant. The example of the minimum con-
ıst calculated further back has been entered into Fig. 14. A comparison
Figs. 14 and 13 reveals that the deviations in density cause very much
ss contrast than the osmium staining; this is true even where a growth
density to double its value ($\Delta\varrho/\varrho = 1$) occurs.

SUMMARY

Recent advances in preparation techniques for electron microscopy open
) possibilities for quantitative cytochemical and histochemical work. A
eatment of the theoretical aspects is given aiming at actual work on routinely
oduced sections of biological material. The contrast in pictures from ma-
rial treated with heavy metals or electron stains is treated in a special sec-
)n, as are the possibilities of "weighing" object details by means of electron-
icroscopical procedures. For practical purposes two nomograms are pro-
ded to facilitate the calculation of dry weight, section thickness and specific
avity from contrast or vice versa.

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verkusen, for their continuous interest and aid.

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SUBMICROSCOPIC ORGANIZATION OF THE MITOCHONDRIAL BODY AND OTHER CYTOPLASMIC STRUCTURES OF INSECT TESTIS[1]

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RECENT progress achieved by electron microscopy in the study of the fi structure of mitochondria has renewed the interest in the extraordina changes that this cell organoid undergoes during the spermatogenesis certain invertebrates. Since the last century it is known that mitochond from the seminal cells of insects—after the second maturation division the spermatocyte—assemble in one locus and become fused to form the called mitochondrial body or "nebenkern". This component was discove by von La Valette St. George in 1867 [22] who named it "Nebenkörpe the denomination "Nebenkern" is due to Bütschli in 1871 [4]. Later stud [3, 7, 8, 9, 15, 16] have contributed to reveal the real significance of nebenkern and to differentiate it from other cytoplasmic structures with wh it has been confused in the past [24]. These cytological studies have revea that the mitochondria which have agglutinated to form the mitochondr body, sooner or later differentiate an inner chromophobic and an ou chromophilic region. The latter stains intensely with mitochondrial dy Once the "nebenkern" is constituted, the chromophobic zones form cl areas of vacuolar aspect separated by concentric chromophilic partitions. later stages the chromophile substance diminishes and tends to disappea the moment when the mitochondrial body divides into two along the a of the spermatid. Finally each resulting body elongates to become incor rated to the sheath of the spermatozoon filament.

Beams *et al.* in 1954 [1] made a study of the mitochondrial body of grasshopper with the phase and the electron microscope. With phase mic scopy they confirmed the classical cytological observations describing the early "nebenkern" a clear outer area of vacuolar aspect that gr until it reaches the stage of a concentric lamellar structure. The elect

[1] Work supported by a grant from the Rockefeller Foundation

icroscope observations were unfortunately handicapped by technical arte-cts and the authors were unable to resolve the fine structure of mitochondria id to follow it through the profound changes they undergo to form the iebenkern".

This lack of information regarding the mitochondrial body made us decide publish these observations made in connection with a study of the fine struc-re of the nucleus in the spermatocytes and spermatids of the grasshopper *iplatacris dispar* [6]. This study includes a brief description of other cyto-asmic constituents that are found in these cells and that should be differ-tiated from the mitochondrial body. These observations are by no means haustive and do not intend to follow the different cytoplasmic components roughout the entire spermatogenesis. Instead, we have placed emphasis the fine structure of the mitochondria and on the structural bases of the namic process that leads to the formation and evolution of the mitochond-il body.

TECHNIQUE

The testicular follicles from the grasshopper *Laplatacris dispar* were rapidly excised d immediately immersed in the fixative. A few minutes later the individual follicles re dissected and treated with fresh fixative for 2 to 6 hours. The fixing fluid is nilar to the standard solution of Palade [11], with small modifications. The solution is prepared from equal parts of 2 per cent osmium tetroxide, veronal-acetate buffer pH 7.4 and of Ringer with a concentration of $CaCl_2$ of 0.5 per cent. After the fixa-n and dehydration each follicle was cut into three segments; each one corresponding about $\frac{1}{3}$ of the total length of the follicle. This was done in order to obtain a better alysis of the different generations of cells that are superimposed from the apex, iere the spermatogonia lie, to the bottom where the mature spermatozoa accu-ılate. Thin sections were made with a special ultramicrotome based on the model Porter and Blum [18] but equipped with thermal advance. The thickness of the tions was appraised by the interference colors. In some cases thick (0.5-1 μ) tions were observed with the phase microscope after removal of the embedding th acetone or xylene. The electron micrographs were obtained with an RCA. ctron microscope model EMU 2C provided with a well compensated polar piece d apertures in the objective, projector and condenser. The original photographs re obtained at magnifications ranging between 6 and 12,000 × and enlarged otographically to 60,000 × or more.

OBSERVATIONS

Distribution and fine structure of the mitochondria in the early spermatid.— hile in the primary spermatocyte, mitochondria are distributed more or s at random, a remarkable change has occurred in the very early spermatid.

Practically all the mitochondria of the cell are localized in a definite regi of the cytoplasm near the nuclear membrane. An electron micrograph co responding to this first period shows circular, oval or more elongated profi of sectioned mitochondria as observed in cells in which there is a rando orientation of the condriome (Fig. 1). Observation of many electron micr graphs including some of serial sections indicates that in this material mi chondria are elongated bodies with a width ranging between 200 to 500 m with a mean of 312 mμ (61 measurements). Among these profiles there some that are V-shaped or show a much more bizarre outline. The frequen of these forms and their progressive increase in number indicate that th result from the fusion of two or more mitochondria. That this is a real fus and not simply the result of the attachment of one mitochondrion to anot is demonstrated by the continuity of the matrix and mitochondrial membr (Fig. 1). Interspersed among the mitochondrial profiles there are some t vesicular and tubular-like structures corresponding to the endoplas reticulum [17], some few dense particles [13], and a cytoplasmic matrix low electron density.

The internal structure of the mitochondria corresponds to that now r ognized for this organoid in all kinds of cells [10, 12, 20, 21]. One may tinguish a limiting membrane of about 110 Å which in some micrograp appears to be double, the mitochondrial matrix, and the crests (Fig. Observation of the different profiles of mitochondrial crests shown in section tends to indicate that they are not lamellar as in most cell types, thin tubular-like projections of the internal membrane with a diamete about 220 Å (Figs. 1, 6). These fine tubular projections have a free en the mitochondrial matrix. This fact can be observed exceptionally wel sections of the mitochondrial body (Fig. 6). Some other details of the structure of the crests and of the matrix will be described later.

Agglutination of mitochondria.—After the above-mentioned change in distribution of the mitochondria has occurred, they converge further they become agglutinated (Figs. 2, 3, 4). Progressively all the mitochon of the cell collect into a single body irregularly shaped at first, but ten to become spherical or oval. At this early stage the boundaries of the i vidual elements are still defined and the mitochondria maintain their orig size. The mitochondrial membranes are particularly dense and progress their profiles tend to resemble a jig-saw puzzle.

Changes in the fine structure of the mitochondrial body.—Once the aggl tion of all the mitochondria has occurred an intense process of remode of the mitochondrial body takes place. The adhesion of the mitochon

embranes continues, but at the same time the fusion of the individual mponents seems to proceed at a much faster rate. At a stage of full develop-ent the "nebenkern" appears as a huge oval body near the nucleus with a ameter of about 5.5 by 3.2 μ (Fig. 5). The section shows in the outer region ge mitochondrial profiles of about 1 to 1.5 μ in diameter that are connected rectly with the small inner mitochondria. Although no definite decision is ssible without studying the complete serial section, one obtains, by observ-g many electron micrographs, the impression that the entire mitochondrial dy is now formed by a single mitochondrion subdivided by a labyrinth of egularly concentric membranes in a jig-saw fashion. In the outer region large profiles are due to an increase of the mitochondrial matrix which w separates wide open the mitochondrial crests emerging from both sides gs. 5 and 6). The observation that the electron density of the matrix re-ains unchanged tends to indicate that the increase in size of the outer pro-s is due not to swelling but to the fusion of many of the original mitochon-a. Furthermore the fact that the profiles observed in the inner part of the dy are of a smaller diameter than the primitive mitochondria (mean dia-ter of 140 mμ, 32 measurements) tends to indicate that probably the matrix s been pushed out from the inside toward the periphery. This concept plies the communication between the inner and outer part of the mito-ondrial body and the flow of matrix from the interior of the "nebenkern" ard the periphery.

Some details of the fine structure of a well-developed mitochondrial body visible in Fig. 6. The mitochondrial membrane is definitely double with otal thickness of 110 Å which includes two 30 Å membranes of high elec-n density and a lighter space of about 50 Å. It is interesting to note that membranes of the adherent mitochondrial profiles are maintained parallel each other with a rather even spacing of about 240 Å. This space has an tron density that is higher than the cytoplasmic matrix, as if some material re present in it. The total distance separating the matrix from one mito-ondrial profile to the adjacent one is thus of about 460 Å. The mitochon-al tubule-like crests are seen to be in continuity with the inner mitochon-al membrane. The diameter of the tubules is about 220 Å, 160 Å of which respond to the inner lighter core, the surrounding membrane being about Å thick. It is interesting to see that some of these tubular crests have bal-ned in some regions making a vesicular space. In high resolution micro-phs it is possible to demonstrate that the mitochondrial matrix has a y fine (below 40 Å) unoriented filamentous component evenly distributed l filling the entire space (Fig. 6).

Division and further evolution of the mitochondrial body.—Our electro micrographs do not permit of a complete reconstruction of the last stages evolution of the mitochondrial body. It is, however, possible to demonstra that the increase in size of the outer mitochondria continues at the expense the inner region mainly composed of mitochondrial membranes. There probably a process of membrane flow (see discussion) from the inner in the outer region and possibly also the partial reabsorption of the membrane Because of this evolution the mitochondrial body loses its distinct initi differentiation into an inner and an outer region. These changes are probab connected with the division of the entire "nebenkern" into two large ov bodies (Fig. 8). In this figure it is possible to observe the cross section of t axial filament of the spermatid that lies in between the bodies while in Fig. it could be observed on one side of the original "nebenkern".

The mitochondrial nature of these two bodies may be difficult to ascert at the first glance, but the presence of a few mitochondrial crests permit sure identification. The profiles of lower electron density found inside the t mitochondrial bodies are difficult to interpret on the basis of present e dences. Fig. 7 illustrates the beginning of the lengthening of these mitochondr bodies in order to form the sheat of the tail. A very large and elongated bo is seen occupying the space between the nucleus and a centriole. The mi chondrial nature of this body is still recognizable in spite of the fact t the number of mitochondrial crests seems to have diminished and the f that have been observed have a vesicular appearance.

Brief description of other cytoplasmic components.—The electron mic scope study of this material allows a clear distinction of the mitochond body from other cytoplasmic constituents. A brief description of the centr

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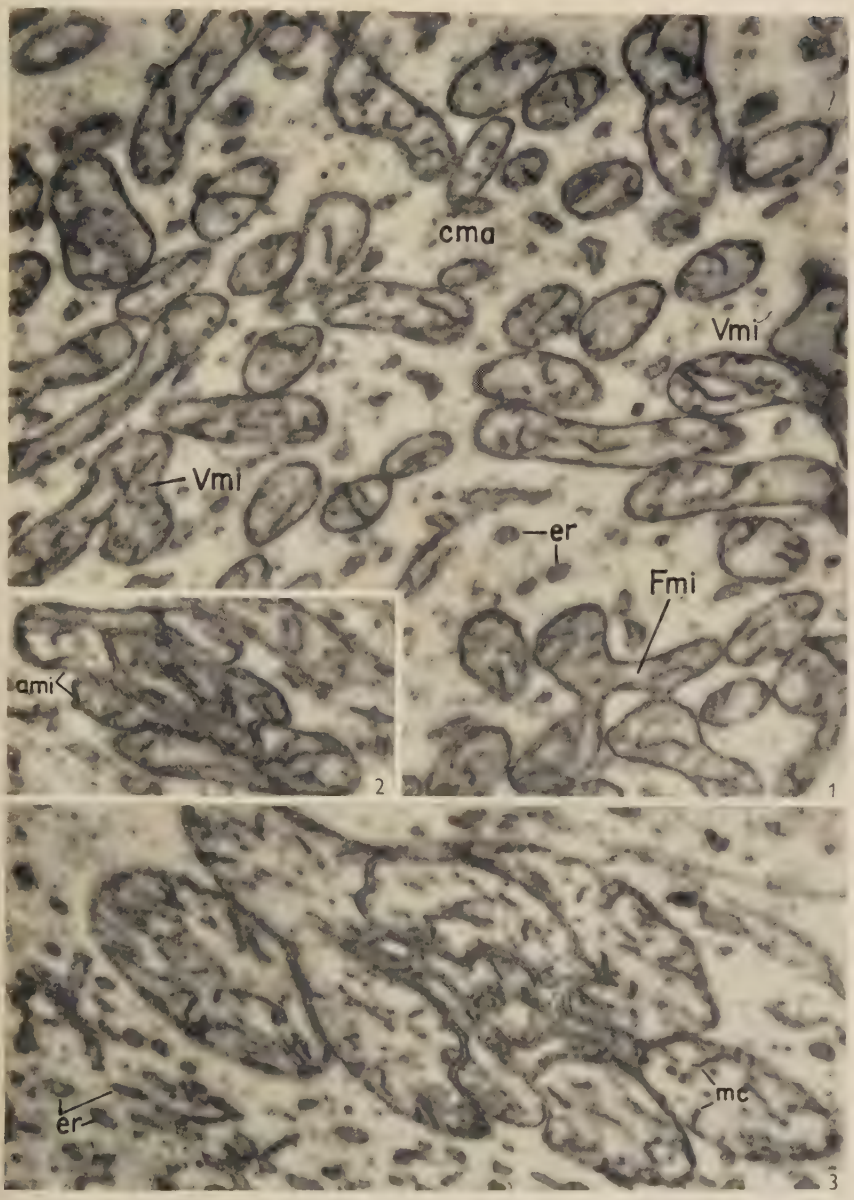
Abbreviations

Af, axial filament; *aer*, agranular endoplasmic reticulum; *ami*, agglutinated mitochon *Ce*, centriole; *cm*, cell membrane; *cma* cytoplasmic matrix; *dp*, dense particle; *er*, endopla reticulum; *Fmi*, fused mitochondria; *G*, Golgi body; *ger*, granular endoplasmic reticulum mitochondrial crest; *mf*, filaments of the mitochondrial matrix; *mib*, mitochondrial body; mitochondrial membrane; *mm*, mitochondrial matrix; *N*, nucleus; *Vmi*, V mitochondria.

Figs. 1–10 are electron micrographs from thin sections of seminal cells of the grasshopper *La cris dispar.*

Fig. 1. Area of the cytoplasm of an early spermatid showing the localization of mitochon Some of them have fused showing a V or a more bizarre shape (see the description in the t × 22,000.

Figs. 2 and 3. Several mitochondria in the process of agglutination. The deformation o mitochondrial profiles by the adhesion indicates the plasticity of this organoid. It is clearly that the mitochondrial crests are tubule-like. × 20,600.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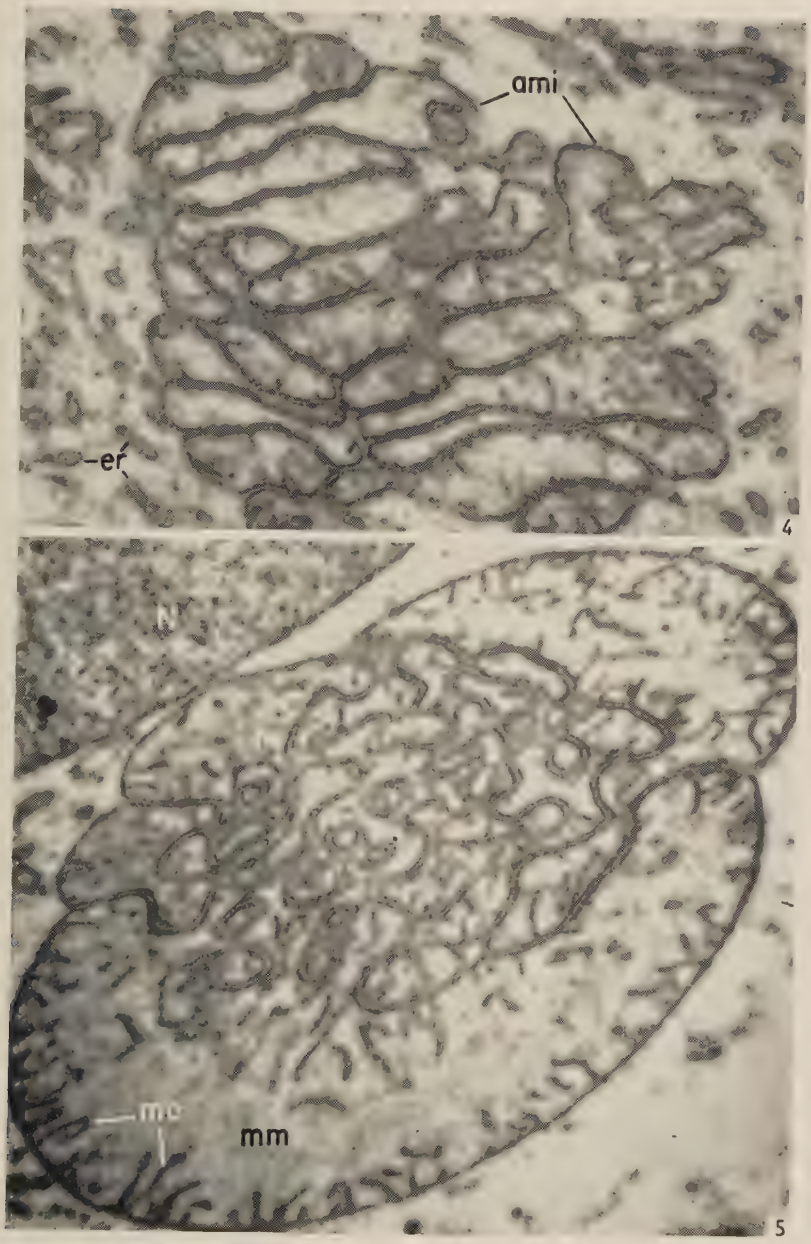
has already been advanced [6]. It appears as a round or oval-shaped mass 1–2 μ with very intense electron density. This mass is composed of very den granules (the densiest component observed in this tissue) of about 160 Å diameter tightly packed in the midst of a dense intervening matrix. In son cases as in Fig. 7 there are areas of low electron density and without granul within the mass of the centriole, but this does not occur constantly. TI centriole shown in Fig. 7 is probably the distal one lying at this stage in t spermatid cytoplasm. It is interesting to see that it is surrounded by a crow of vesicular and tubular material of the endoplasmic reticulum. The rel tionship of the proximal centriole with the nuclear membrane and the stru ture of the nucleus has been described [6].

The Golgi bodies [5] can be clearly distinguished from the mitochondr body by their compact structure made of tightly packed tubular and vesicul profiles (Fig. 10). The distribution of the so-called endoplasmic reticulu [17] showing definitely differentiated areas is of considerable interest. TH system is composed of tubular and vesicular profiles that pervades loos the entire cytoplasm of spermatocytes and spermatids (Figs. 7, 9 10). The profiles are generally composed of a limiting membrane and have a conte that is denser than the cytoplasmic matrix. Most of the endoplasmic reticulu has smooth outlines and is "agranular" in the sense that it does not sh the attached granules of Palade [13]. However, in certain definite regions the cytoplasm, frequently near the nucleus, one may find a special differenti tion of the endoplasmic reticulum consisting in a superposition, at regu intervals, of flattened cisternae. In these regions the membrane has a rou surface because of the presence of the attached granules. However, continuity of this granular endoplasmic reticulum with the more comm agranular type is clearly apparent (Fig. 9).

DISCUSSION

The study of thin sections of cells with the electron microscope has, amo other important findings, resulted in the identification of a specific s

Fig. 4. Numerous mitochondria that maintain their original size and limit are agglutinated a huge mitochondrial body. This is irregular in shape and has the aspect of a jig-saw pu Observe that the mitochondrial membranes are separated by an even space. × 22,600.
Fig. 5. Mitochondrial body in full development. The intense process of remodelling that occurred is apparent by comparing with Fig. 4. There is an inner zone with small profiles co tuted mainly by mitochondrial membranes and an outer region with large profiles rich in m chondrial matrix. The continuity between the outer and the labyrinthic inner region is appa (see further description in the text). × 22,000.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microscopic organization for mitochondria. In fact it is possible to obser in each mitochondria a double limiting membrane, a system of internal ridg (or mitochondrial crests) as folds of the internal membrane, and a matr [10, 12, 13, 20, 21]. This structural pattern can be found, with minor vari tions, in all animal and plant cells and in protozoa, and thus permits a cle morphological identification of this organoid and its derivates as in the ca of the mitochondrial body. In the old literature some questions were rais about the nature of the nebenkern particularly because, according Wilson [24], this term was often misapplied to other structures such as t acroblast, the secondary nuclei of the oocytes, the spheric masses of bas phile substance, the ergastoplasm of the pancreas, or other secretory cel However, in 1900 Meves [9] already showed that the nebenkern does n arise from spindle fibres as was formerly believed, but from the aggregati of mitochondria.

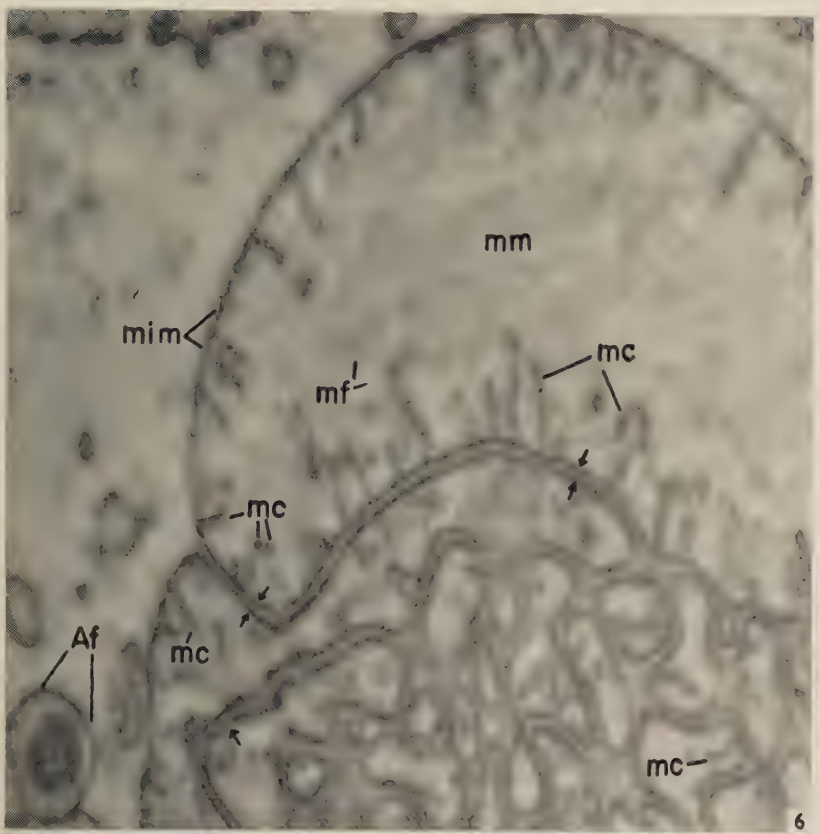
In their electron microscope study on the nebenkern Beams *et al.* [although recognizing its mitochondrial orgin and nature, were unable to esta lish a relationship between its lamellar organization and the fine structu of mitochondria. In our electron micrographs this parallelism can be perfec demonstrated and the fundamental structural pattern of mitochondria c be recognized throughout the complex and profound changes occurr during the evolution of the mitochondrial body.

Two intimately related mechanisms seem to be at work in the formati of the nebenkern—the agglutination and the fusion of mitochondria. T study of different living cell types in tissue culture has permitted to obse the active changes of shape and size that mitochondria may undergo w extreme rapidity. Agglutination and probably fusion of mitochondria pr ably occur in many cell types, but in the spermatids of the insects th phenomena reach a maximum development.

From the observation of the electron micrographs it is impossible to est lish which is the mechanism involved in the agglutination. The mitochon may be ordinarily drawn together by cell motion or by some other unkn force, but whatever is the cause there must in this case be a change in

Fig. 6. High resolution electron micrograph of a fully developed mitochondrial body (see detailed description in the text). Arrows indicate regions in which the double mitochon membrane is clearly observed. Note that the crests are tubule-like projections of the inner m chondrial membrane. × 38,600.

Fig. 7. Cytoplasmic region of a late spermatid showing an elongated mitochondrial body an centriole surrounded by a crown of endoplasmic reticulum. The macromolecular structure o centriole is clearly visible (see description in the text). × 38,600.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properties of their surface membrane. Some kind of stickiness probab develops that holds the mitochondria together at one point. Once this h happened the adherence of the mitochondrial membrane progresses pushi out the intervening matrix and the endoplasmic reticulum. That adhesio forces are involved in this process is also suggested by the deformation und gone by the individual mitochondria to occupy a minimum of space with the mitochondrial body. This change in the properties of the mitochondri membrane leading to agglutination may be related to the coating of the surfa by some special component. This assumption is favored by the fact th within the nebenkern the mitochondrial membranes are separated by remarkably constant distance of about 240 Å and that this space is occupi by a component that shows some electron density.

The mitochondrial membranes with this interposed component and t crests probably correspond to the chromophile substance of the cytologi literature [24] while the mitochondrial matrix represents the chromopho region. If this is the case, one may understand that, at the low level of reso tion of the optical microscope, the nebenkern has the appearance of a c centric structure with clear areas of chromophobe substance separated thin partitions of chromophile material. The fact that the membranes beco reduced during the process of fusion of mitochondria also explains w the chromophile substance diminishes and disappears when the mitochondr body divides.

The complex changes undergone by the mitochondrial body during evolution illustrate in an interesting way a dynamical structural phenomen occurring at a submicroscopic level. We have here another example of me brane flow as it has been postulated for other membrane systems of cytoplasm and of the nuclear and cell surface [2, 14], and which has b considered by Bennett [2] as actively engaged in the transport of substan within the cell. Not only is there a flow and fusion of the mitochondr membranes but, as can be inferred from our measurements, the ma probably flows from the inner to the outer partitions of the mitochondr

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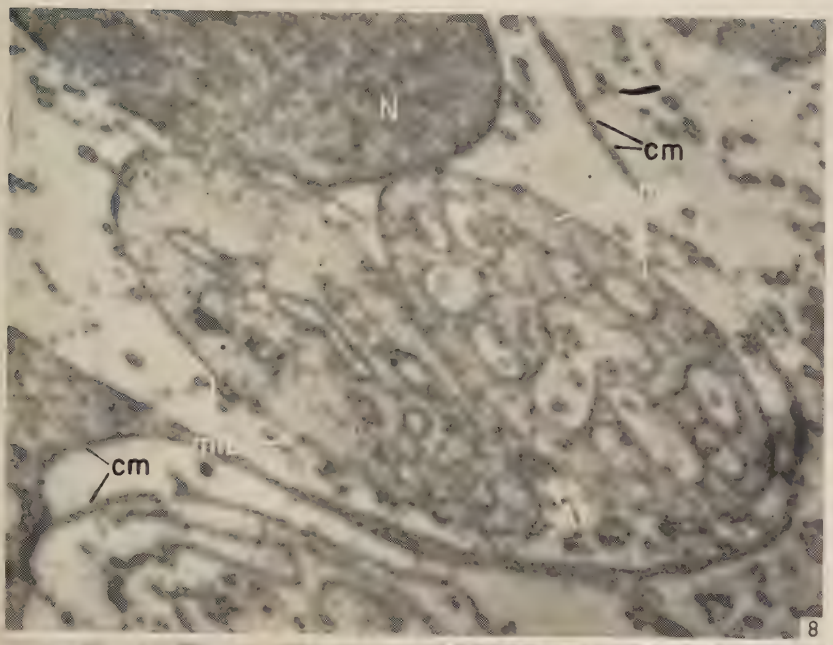
Fig. 8. Cytoplasm of a late spermatid showing the two mitochondrial bodies resulting from division of the former one. The amount of mitochondrial membrane has diminished and t are some clear areas of difficult interpretation. Between the two bodies passes the axial filam × 19,200.

Fig. 9. Region of the cytoplasm of a spermatocyte near the nucleus showing a differenti region of the nuclear membrane. The continuity between the granular endoplasmic reticu provided with dense particles and the agranular reticulum is clearly visible. × 32,000.

Fig. 10. Area of the cytoplasm of a spermatocyte showing side by side a Golgi body and of the granular endoplasmic reticulum. × 27,200.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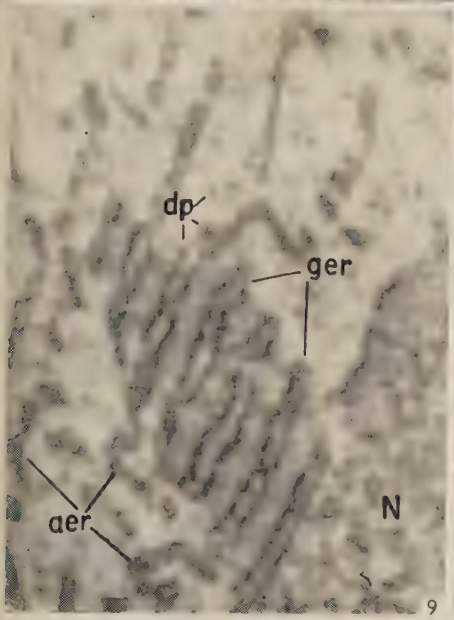
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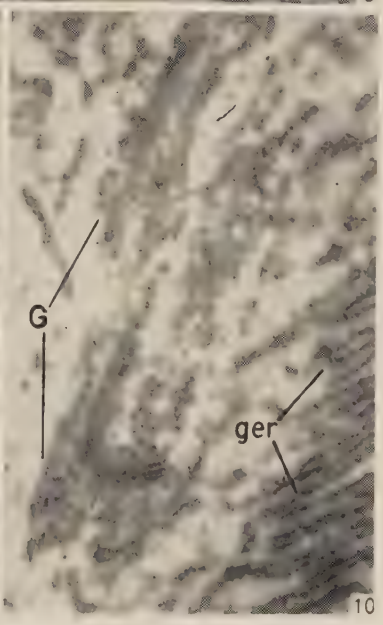
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body. This flow of the mitochondrial matrix is a clear demonstration that th mitochondrial crests do not constitute complete partitions of the mitocho drial cavity as was demonstrated by Palade [10, 12] but discussed by Sjöstra [20, 21]. In this material the mitochondrial crests are tubule-like projectio of the inner membrane that end freely in the mitochondrial cavity. Th fact is particularly clear in the large peripheral cavities of the mitochondri body. Another interesting finding is the fact that the mitochondrial matr is not amorphous but shows a fine filamentous macromolecular compone evenly distributed and with a random orientation. The possible relationsh of this component with the soluble proteins and enzymes known to be prese in mitochondria (see [19]) should be investigated.

SUMMARY

The fine structure of mitochondria and the structural changes leading the formation and evolution of the mitochondrial body or "nebenkern" the spermatid of insects have been investigated in the grasshopper *Laplatac dispar*. Each mitochondrion possesses a double mitochondrial membra tubule-like crests and a mitochondrial matrix. In this material it is clea demonstrated that the crests are folds of the inner mitochondrial membra that end freely in the mitochondrial matrix.

In the early spermatid the localization and the early fusion of so mitochondria in one region of the cytoplasm were observed. This perioc followed by the approachment and agglutination of all mitochondria form the mitochondrial body. The probable mechanisms that may be volved in this agglutination are discussed.

This stage is continued by an intense process of remodelling of the m chondrial body, leading to a differentiation of an inner region made of fu small mitochondrial profiles and an outer one of large profiles filled w abundant mitochondrial matrix. The inner and outer profiles are in am continuity. An active process of flow of the mitochondrial membranes of the matrix is postulated. The mitochondrial matrix shows the prese of a fine macromolecular filamentous component. This phenomenon is other indication of the fact that there is a continuity in the mitochond cavity and that there are no complete partitions of it. A labyrinthic even sp 240 Å wide, occupied by a material of weak electron density separates fused mitochondrial membranes. The intense remodelling of the mitoch drial body leads to a diminution of the membranes and finally to the divis of the "nebenkern" into two bodies. In later stages the "nebenkern" is dr

o an elongated body that still maintains a modified but characteristic tochondrial structure.

The fine structure of other cytoplasmic components of the seminal cell, luding the centrioles, Golgi bodies and endoplasmic reticulum, is described. e endoplasmic reticulum is mainly agranular but shows differentiated gions with attached granules (granular endoplasmic reticulum). Both types reticulum are in direct continuity.

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A STUDY ON ENDOCHONDRAL OSTEOGENESIS IN TISSUE CULTURE

A PRELIMINARY REPORT

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Bone and cartilage have been the object of intensive investigations durin recent years. However, the osteogenetic capacity of the isolated long-bon cartilage has not been thoroughly investigated. Since the classical works H. Fell on osteogenesis only sporadic studies have been published on th subject, and our modern concepts on the procedures of endochondral o teogenesis are still open to discussion. The present investigation was undertak in order to study endochondral osteogenesis during embryonic life from morphological, biochemical and crystallographic standpoint.

It is the purpose of this paper to give a report of the results so far o tained from a series of explantations in tissue cultures of well-differentiat epiphyseal cartilage taken from fowl embryos and deprived of its co nections to perichondrium and other surrounding tissues.

MATERIAL AND METHODS

The watch-glass method, as described by Fell and Robison [4], was the tissue c ture method used throughout. At the present time 569 explants have been studi Of these 303 were grown in a solid medium, composed of equal parts of cock plas and embryo extract, diluted with Tyrode's salt solution containing 4 per mi glucose [8], in a proportion one part embryo extract to three parts Tyrode's solutio while 266 explants were grown according to the method described by Chen [using a fluid medium composed of one part embryo extract diluted with an eq amount of Tyrode's solution, containing 4 per mille glucose, and three parts ho serum. The medium was filtered in order to exclude any cellular influence on explants. The explants were grown on floating lens paper (Zeiss Vinkel). To impro calcification a Tyrode's solution with calcium glycerophosphate (0.2 per cen according to Fell and Mellanby [3], was used in the treatment of certain ser

The explants were, in all but a few cases, derived from 12-day-old chick embry Ten explants were obtained from 14-day-old, ten from 13-day-old, twenty fr 11-day-old and thirty from 10-day-old embryos. The cartilage from the lower a

e upper tibial and the lower femoral epiphyses were used. The preparation of the plants was performed under a dissecting microscope. The long bones of the lower tremities were freed from their connections with the surrounding soft parts. The riosteum was stripped off and taken away together with the perichondrium covering e ends of the bones and the articular cartilage. The thin perichondral bony cylinder, closing the cartilaginous cones, was then opened by a longitudinal incision and the ne layer was pulled away by gentle scraping. The distal, i.e., the diaphyseal, end the cones usually perforated by medullary tracts and vessels, was cut away. e proximal part, 2–4 mm long and up to 2 mm wide, a flattened cylindric formation, llowed longitudinally when lower tibial and femoral epiphyses were used, was planted after repeated irrigations and washings in Tyrode's solution.

The explants were transferred to fresh media every third day, when a solid medium s used. Explants grown in fluid media were subcultured every fourth day.

In order to check the efficiency of the dissecting technique for removing all nnants of the surrounding tissues, 40 explants, prepared in the way described, re serially sectioned at 10 μ intervals and the sections, about 3000, were studied. none of them were periosteal, perichondral, bony, medullar or vascular tissue nnants found. Meanwhile in a certain number of the sections isolated cells or small ups of cells were observed at the periphery having no morphological similarities typical chondrocytes.

The observations described in the present paper are based on explants grown in sue cultures for different periods up to about 50 days.[1]

RESULTS

The morphological study of the material was based partly upon fibroblastic tivity and areal growth of the explants, partly on histological sections.

The fibroblastic activity was studied in explants grown in solid media. tive outgrowth of cell appeared in cartilage explants much later than in plants of other tissues. As a rule, very weak signs of outgrowing cells ap- ared on the 3rd to 6th day and a typical halo was very seldom formed be- re the 14th day of explantation, a fact which has been stated earlier by her authors.

The areal growth was followed by projection microscopy and tracing of e projected outline of the explants at 5- to 7-day intervals. By aid of a plani- eter the total area of growth was determined. The method is open to many jections but is commonly used, especially when large series are to be llowed. The percentage increase of the area is demonstrated in Fig. 1, where e evolution of the areal growth in different series is given. Solid media were ed in all these series.

The maximal increase of the area was observed on the 40th to 50th day. ne results here obtained differ notably from those of other investigators. The

[1] The results reported in this paper have not been treated statistically.

difference is probably due to the fact that they used cartilages derived fro younger embryos. The same difference was also observed in the prese series, in cases in which the epiphyseal cartilages from younger embry were explanted and grown in solid media (see Fig. 2). The results show in this figure are based on a rather small number of measurements.

As to the rate of the areal growth, a steep rise was observed in all the curv during the first week of explantation, the increase became less marked longer time intervals. In all the series, a remarkable decrease in the are

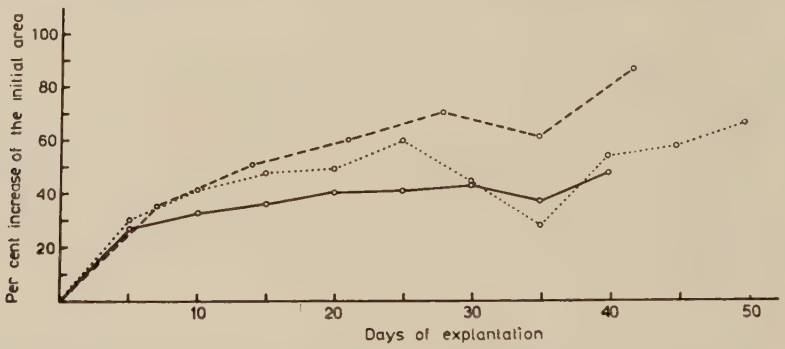


Fig. 1. The percentage of the areal growth in explants grown in solid media. Each curve de onstrates the evolution of the areal growth in one series of explants.

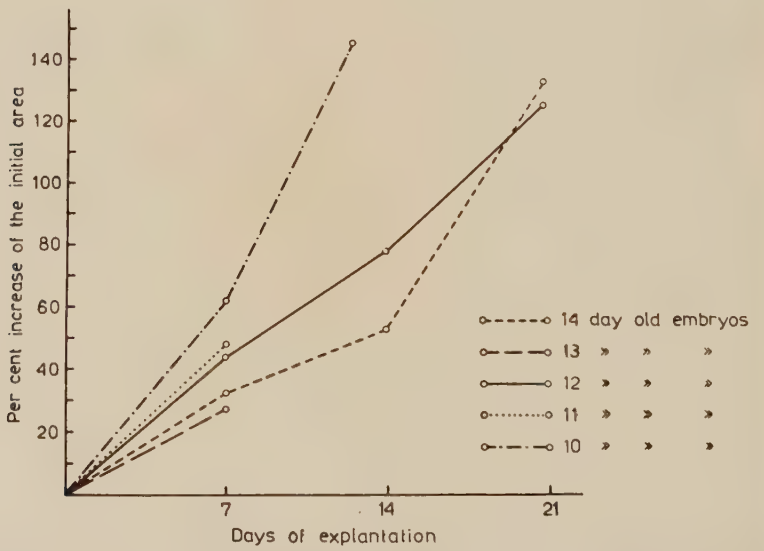
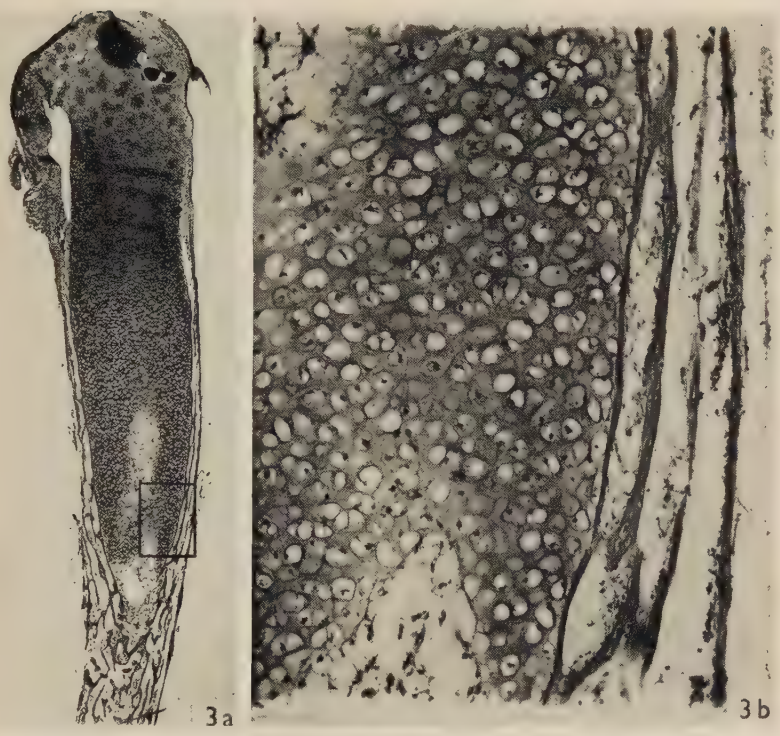


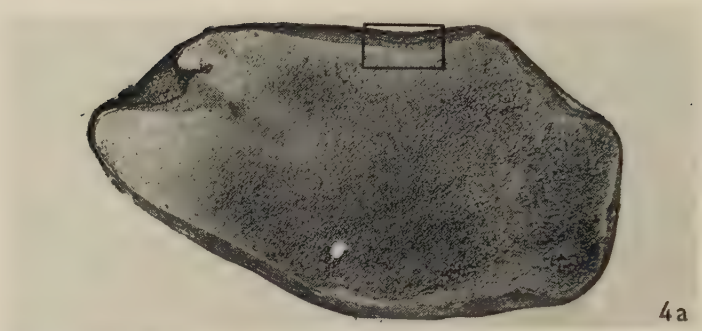
Fig. 2. The percentage of the areal growth in explants derived from embryos of different and grown in solid media.



g. 3*a*. Section through the upper femoral epiphysis of a 12-day-old chick embryo (× 24). ıe perichondrium covers the upper part of the bone. The epiphyseal cartilage cone is eroded · medullary tracts in its diaphyseal end. The three layers of cartilage cells, as described by Fell, e visible. In the diaphyseal part of the picture, cortical perichondral bone is found. This expands wards the epiphyseal end of the bone and forms the bony cylinder covering the cartilage cone. ıe bony cylinder extends towards the junction of the perichondrium and periosteum.
ig. 3*b*. The inset at high magnification (× 250). Periosteum, cortical bone and cartilage are sible.

owth was observed between the 30th and 40th day of explantation, a fact hich could not be explained in a satisfactory way. It was, however, observed ıat many explants, especially the longer ones, became curved on their ngitudinal axis. The middle part of the explants thus raised from the clot · paper surface. The tracings obtained after projection microscopy in such ıses showed a decrease in area. This observation could to some extent ex-lain the fall in the curves of the areal growth. The deformation of the ex-lants was, however, restricted to the first weeks or even in some cases to ıe first days of explantation.

Histological observations.—Histological sections were made on 81 explants fter explantation of 1 up to 53 days. After fixation in a 10 per cent formalde-



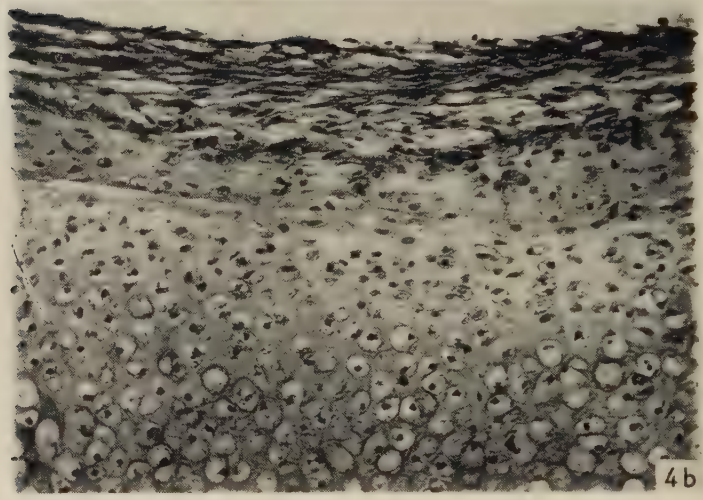
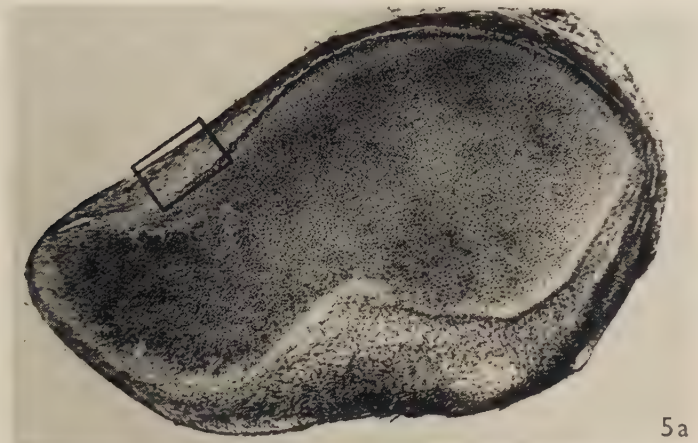
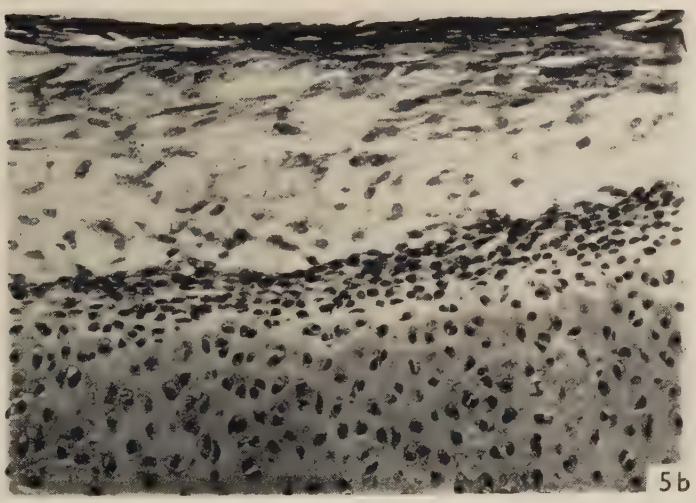


Fig. 4*a*. An explant derived from the upper tibial epiphysis from a 12-day-old chick embryo a explanted in a solid medium for 18 days (× 24). In this stage of explantation the perichondr layer has developed completely and covers the entire surface of the explant.
Fig. 4*b*. The inset at high magnification (× 200). A region of degenerating cartilage is seen tween the well developed perichondral layer (lower) and the healthy cartilage.

hyde solution, the explants were sectioned at a thickness of 5 μ and wo stained with eosin–haematoxylin. Bock's [9] and alizarin red S special stai were also used in some cases in order to detect the deposition of calcium sal The following changes were observed in the sectioned material. In ea stages of explantation, 5 to 10 days, the presence of a fibrous tissue appear consisting of oblong cells with small nuclei. At first the fibrous tissue w localized in the middle part of the explants, but it soon expanded towa the ends and at about the 20th day it enwrapped the whole explant a capsule. This layer shows a remarkable similarity to the perichondrium





ig. 5*a*. An explant derived from the upper tibial epiphysis from a 13-day-old chick embryo id explanted in a solid medium for 39 days (× 29). A thick perichondral layer and regions of reosseous tissue are seen.
ig. 5*b*. The inset at high magnification (× 310). The perichondral (lower) and the cartilaginous pper) layers as well as the layer of preosseous tissue are seen.

ie normal bone as it it seen in Figs. 3 and 4. The cells of the perichondral iembrane probably originated from the outgrowing fibroblasts.

At a later stage of explantation, 20 to 40 days, certain parts of the explant ained metachromatically, usually in the middle part of the cartilaginous ssue, beneath the perichondral layer. Signs of degeneration of the cartilage ppeared at the same sites, followed by cytolysis and caryolysis. In this

way a narrow marginal space occurred which later became filled with cell derived either from the marginal chondrocytes or from the perichondra layer. A zone of small flattened cells was thus formed on the 20th to 40th day of explantation. The new tissue, Fig. 5, strikingly resembled bone tissu as seen in sections of normal bones (Fig. 3).

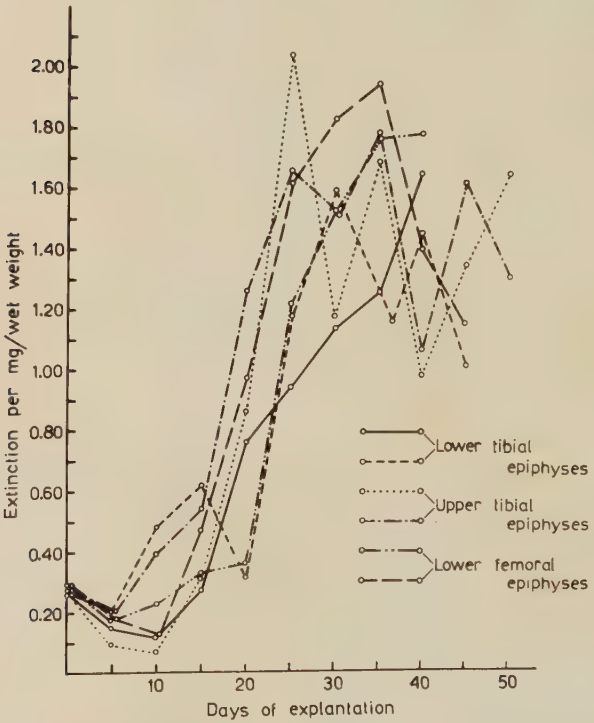


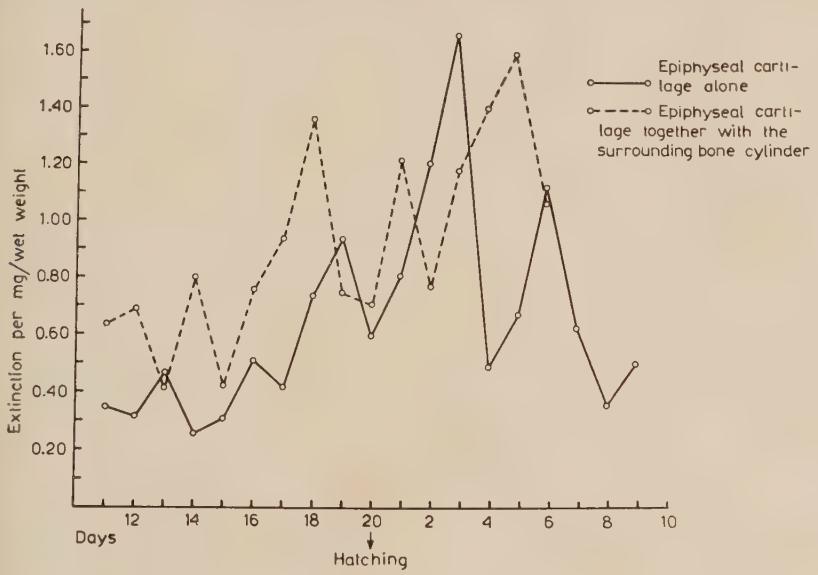
Fig. 6. The alkaline phosphata activity in material grown in sol media.

Every attempt to identify deposition of calcium salts by special stains w unsuccessful in explanted material grown in a solid medium up to 50 day

The biochemical study of the present material consisted in determinatio of different enzymes, alkaline and acid phosphatase and phenolsulfatas and of chondroitin sulfate.

The alkaline phosphatase activity was determined according to the meth described by Binkley *et al.* [1] with slight modifications. Each explan after weighing, was homogenized in a glass homogenizer in the presence 1 ml distilled water, saturated with chloroform. 0.005 *M* di-sodium phen phosphate in a veronal buffer of pH 9.2 was used as substrate. To activa the enzyme, $MgCl_2$ was added to the substrate. The time of incubation w

hours at 37°C. When the activity began to be very high, the time of incubation was limited to 3 or even to 1 hour and then the result was multiplied by or 3 respectively, as the enzyme reaction–time curve was linear in the nge used. The results were read in a Spekker's photometer using an Ilford lour filter, spectrum red 608. Determinations of the alkaline phosphatase



. 7. The alkaline phosphatase activity *in vivo*.

tivity were performed on explants grown on a solid medium. The results tained are given schematically in Fig. 6.

In an attempt to investigate the alkaline phosphatase activity *in vivo* terminations were made on fowl embryos and young chicks of different es after hatching. The upper tibial epiphyses were used. Determinations re performed (*a*) in the epiphyseal cartilage cone dissected free from all surrounding connections and (*b*) in the epiphyseal cartilage cone together th its surrounding bony cylinder. The results referred to here are based a relatively limited number of determinations and are given schematically Fig. 7.

After a decrease from the initial 0-day values, during the first 5–10 days explantation—a period corresponding to the higher increase of the areal owth—the alkaline phosphatase activity in explanted material showed a ep rise between the 15th and 25th day. This rise corresponds to the appearance of the first histological changes in the mass of the explants. Maximal

values were obtained between the 25th and the 40th day, in which perio preosseous tissue was laid down. A decline started after about the 40th da of explantation. No significant differences in the alkaline phosphatase a tivity in explants obtained from different epiphyses were observed.

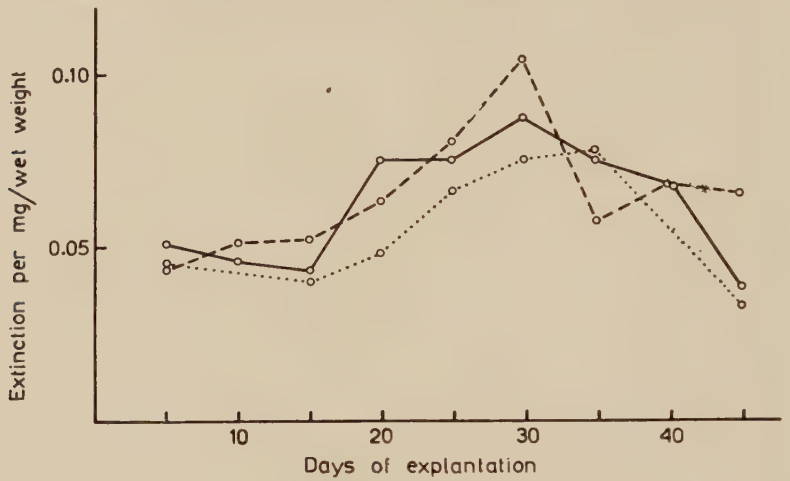


Fig. 8. The acid phosphatase activity in material grown in fluid media.

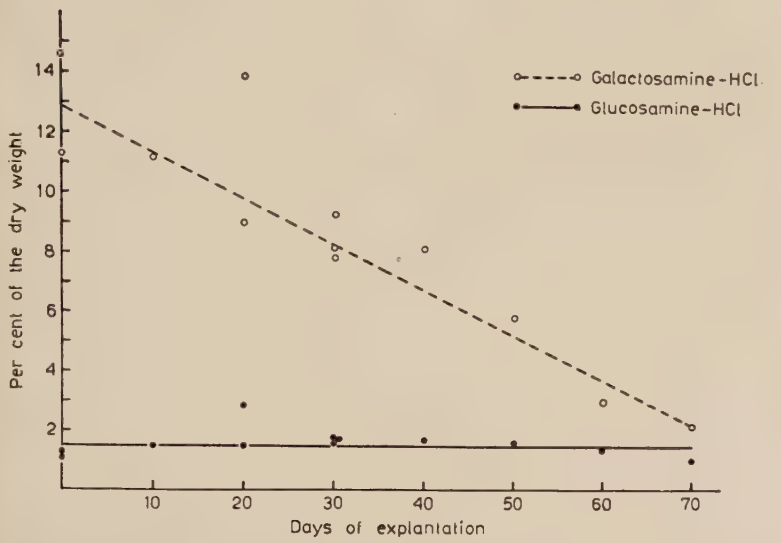
The alkaline phosphatase activity *in vivo* showed an increase proportiona to that observed in explanted material, but the curve obtained showed slower rise with several peaks during the embryonic and early post embryo life.

In order to get some information concerning the possible specificity of t rise of the alkaline phosphatase activity in connection with the proces of osteogenesis, estimations of the acid phosphatase and the phenolsulfat activity were also made.

The phenolsulfatase activity was studied in homogenized epiphyseal co using a 0.005 *M* potassium phenylsulfate in an acetate buffer of pH 5.3 substrate. The technique was similar to that used for the alkaline phosphat [7]. No traces of phenolsulfatase activity could be detected in the epiphys cartilages of 12-day-old fowl embryos before explantation. A study of enzyme at different stages of explantation therefore seemed unnecessary

Determination of the acid phosphatase activity was done according to Bink *et al.* [1]. 0.005 *M* di-sodium phenylphosphate in an acetate buffer of pH was used as substrate. It was found that the acid phosphatase activity sho a progressive rise between the 15th and 40th day with maximal activity on 30th day. The rise was but slight and was not considered to have any defi

ation to the processes of differentiation of the explants. The results obtained m the determination of the acid phosphatase activity are given in Fig. 8. this case determinations were performed on explants grown on fluid edia.



. 9. The chondroitin sulfate values. The drawing of the curves is only hypothetical. 20-day ues are based on infected material.

The chondroitin sulfate was examined by hydrolysing the dry powder of 10 explants of the same age with 6 *N* hydrochloric acid for 8 hours on a iling water bath. The amino sugars—galactosamine HCl and glucosamine l—were then determined by the chromatographic technique of Gardell 6]. The results obtained are based on explants grown on a fluid medium d are demonstrated in Fig. 9. A progressive decrease of the amount of the actosamine HCl was observed from 0 to 70 days of explantation, while at same time the amount of the glucosamine HCl remained stable.

The significance of the changes of the chondroitin sulfate in connection h the osteogenesis has been pointed out earlier [10] and the results of the sent investigation are in agreement with those of earlier observations.

Crystallografic studies.—As pointed out above, attempts were made to detect ns of calcification in advanced explants using special histological stains t the results were negative. Soft X-ray historadiography was also used for same purpose in explants grown in solid media for 35 to 53 days and ated either with the usual Tyrode's salt solution or with that which had

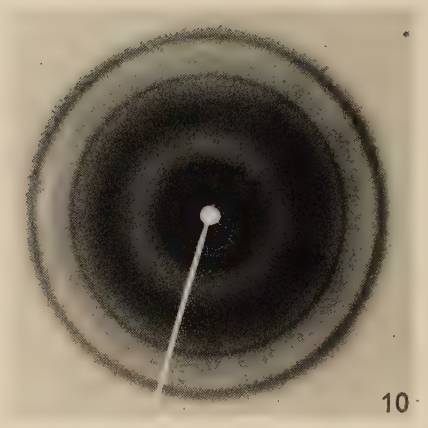


Fig. 10. The X-ray diffraction pattern of an explan grown in fluid media for 60 days.

been enriched with calcium. In one case, after 53 days of explantation, zone with increased absorption was shown by X-ray microscopy. This zon could possibly be explained as a deposition of calcium salts, but the X-r diffraction pattern of the same specimen failed to confirm this concept.

In a series of cultures grown in fluid media, an opaque whitish zone w observed macroscopically, after 50 to 60 days of explantation; it was localiz at the periphery of the diaphyseal part of the explants. X-ray diffraction w applied on one of these, explanted for 60 days. The diffraction pattern o tained showed many diffraction rings characteristic of hydroxyapatite. orientation was observed. The apatite crystallites were of the same order size as those observed in normal bone tissue, i.e. 220–70 Å units.[1]

SUMMARY

Endochondral osteogenesis in tissue cultures has been investigated. T epiphyseal cartilage cones of the long bones of the lower extremities, fr 12-day-old chick embryos *were dissected free from the perichondrium a all the surrounding tissues* and were explanted, using the watch-glass meth either on solid or on fluid media. Observations were performed on mater explanted for as long as 50 days.

Outgrowth of fibroblasts appeared in the cartilaginous explants mu later than it usually does in explants of other tissues. An increase of the a

[1] *Note added in proof.*—A discrepancy between results obtained from material grown in fl media and from material grown in solid media has recently been observed. Thus there is evid that explants grown in fluid media only undergo calcification, whereas explants grown in s media show a partial transformation into preosseous tissue. Furthermore, calcification of expl grown in solid media was demonstrated without simultaneous rise of the alkaline phospha activity. This indicates that calcification may occur in the absence of high alkaline phospha activity.

owth, 50 to 125 per cent of the initial area, was observed. As a rule, the curve the areal growth rose steeply during the first week of explantation and then e increase continued at a slower rate.

Histological sections showed that, at an early stage of explantation, the plants became wrapped in a layer of fibrous tissue having great morphogical similarities to the perichondrium of normal bones. Metachromatic aining, degeneration, absorption of the cartilage and deposition of a preosous tissue followed.

Calcification of the new bone tissue probably takes place after the 50th 60th day of explantation.

The alkaline phosphatase activity showed a steep rise between the 15th id 20th day, with maximal values between the 25th and 40th, and a benning decline after the 40th day of explantation. Acid phosphatase activity owed but a slight rise between the 15th and 40th day.

No phenolsulfatase activity could be detected in the epiphyseal cartilages chick embryos.

Determinations of the chondroitin sulfate showed a progressive decrease the amount of galactosamine HCl between 0 and 70th day of explantation, hile at the same time the amount of glucosamine HCl remained stable.

X-ray diffraction applied on one explant after 60 days of explantation owed a pattern characteristic for hydroxyapatite. The crystallites being the same order of size as those observed in normal bone tissue.

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STUDIES ON THE DIMORPHISM OF *HISTOPLASMA CAPSULATUM*[1, 2]

I. THE ROLES OF –SH GROUPS AND INCUBATION TEMPERATURE

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The roles of sulfur-containing compounds and an optimum temperature in the mycelial (M)–yeast (Y) dimorphism of *Histoplasma capsulatum* have been reported by Salvin [4, 5, 6] and more recently by Pine [2, 3], among others. The phenomenon of dimorphism has been reviewed by Scherr and Weaver [9]. It is generally accepted that, in addition to other requirements, an incubation temperature of 37°C is essential for the growth of the Y phase. One of the aspects of the work here reported concerns conditions under which Y phase growth of this organism can be maintained at temperatures less than 37°C.

Because the human host or experimental animal permits only yeast phase multiplication of *H. capsulatum* (and related dimorphic fungi). Whether Y or M cells constitute the inoculum, it was proposed [7] that Y⇌M dimorphism is probably regulated by biochemical pathways that are mutually exclusive. It should follow from this that conditions which would favor the growth of one phase would also specifically inhibit the growth of the other: this contention gains strong support from the work here reported.

MATERIALS AND METHODS

The following medium was used with the addition of varying concentrations of L-cysteine. All solutions were sterilized by Seitz filtration. The following *basal medium* was used: 0.75 g $(NH_4)_2SO_4$, 0.75 g K_2HPO_4, 0.75 g KH_2PO_4, 0.30 g $MgSO_4 \cdot 7H_2O$, 0.015 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.0065 g $MnSO_4 \cdot 2H_2O$, 1.5 g glucose, 0.1 μ/ml biotin, and 150 ml dist. H_2O.

The inocula of Y phase cells were harvested from blood agar slants incubated for 7–10 days at 37°C and washed twice with sterile saline. Counts of the Y inoculum were made with a haemocytometer, each yeast cell being counted as one.

[1] This study was supported by a grant from the University of Illinois Graduate Research Fund.

[2] A preliminary report of this work was made before the 56th annual meeting of the Society of American Bacteriologists [8].

The mycelial phase was prepared by inoculating glucose broth cultures with Y se cells and incubating for 7–10 days at 25°C. These cells go into a mycelial phase out matting and make harvesting much less difficult. The M phase cells are then hed twice with saline and standardized nephelometrically against the counted Y se suspension.

All experimental cultures were of 5 ml volume and were contained in 15 ml screw test tubes kept tightly closed except when being sampled.

Growth was assessed in a relative manner as (+ + + +)—excellent; (+ + +)—derate; (+ +)—fair; (+)—poor; (+/−)—doubtful; (−)—none. The relative proion of mycelial and yeast phase growth was estimated by microscopic examinaıs of the cultures using wet mounts under coverslips.

RESULTS

Tables I–VIII record the data of growth using varying amounts of Y or phase inocula. These cultures contain no agar. It is clear that with the ɔcula used here, of either phase, there is in general less growth with increase incubation temperature and/or increase in cysteine concentration. Using ɔcula of 10^5 Y cells/ml of medium or less or the equivalent concentrations M cells, no yeast phase growth was found under any of the conditions of mperature or cysteine concentrations studied (Tables I, II, III, V, VI, VII). Using a Y phase inoculum of 10^6 cells/ml of medium it was possible to aintain a preponderant Y phase growth at temperatures less than 37°C the cysteine content of the medium was sufficiently high. Thus (Table IV) ter 7 days incubation at 25°C, 90 per cent of the culture was in the Y phase the 0.004 *M* cysteine concentration; 90 per cent of the culture was in the Y ıase after 14 days at this temperature at the 0.01 *M* concentration. At 30°C cubation temperatures the comparable cultures showed decidedly less Y ıase growth. It is clear here (and consistent with other experiments perrmed during the course of this study) that 30°C is a less optimum tempera-re for the maintenance of Y phase growth than 25°C, other conditions being e same.[1]

With the increase in time of incubation to 21 days there is generally a ecrease in the proportion of Y cells at all temperatures studied (Table IV). ıis might be attributed to the depletion of reduced sulfur in the medium ith the increased age of the culture.

Starting with a Y phase inoculum there is not immediate 100 per cent nversion to the mycelial phase in the absence of cysteine, the rate of this →M conversion being slower at 37°C than at 25° or 30°C. Note (Table IV)

[1] After this work was done, Dr. Leo Pine of the National Institutes of Health informed us that too had been able to maintain Y phase growth at incubation temperatures less than 37°C.

TABLES I–XX. The effect of cysteine on the dimorphism of *H. capsulatum*.

°C	L-cysteine (*M*)	Growth	Phase (%) M	Y	Growth	Phase (%) M	Y	Growth	Phase (%) M	Y
1	2	3			4			5		
		TABLE I—*Inoculum 10³ cells per ml medium, Y phase, no agar.*								
25	.000	–			+	100	–	+ +	100	–
	.001	–			–			–		
	to									
	.05	–			–			–		
30	.000	–			+	100	–	+	100	–
	.000	–			–			–		
	to									
	.05	–			–			–		
37	.000	–			–			–		
	to									
	.05				–			–		
		TABLE II—*Inoculum 10⁴ cells, Y phase, no agar.*								
25	.000	–			+ +	100	–	+ +	100	–
	.001	–			–			–		
	to									
	.05	–			–			–		
30	.000	–			+	100	–	+	100	–
	.001	–			–			–		
	to									
	.05	–			–			–		
37	.000	–			–			–		
	to									
	.05	–			–			–		
		TABLE III—*Inoculum 10⁵ cells, Y phase, no agar.*								
25	.000	+/–			+ + +	100	–	+ + +	100	–
	.001	+/–			+	100	–	+ +	100	–
	.004	–			+/–			+	100	–
	.007	–			–			–		
	to									
	.05	–			–			–		
30	.000	+/–			+ +	100	–	+ +	100	–
	.001	+/–			+	100	–	+	100	–
	.004	–			+/–			+	100	–
	.007	–			–			–		
	.01	–			–			–		
	.05	–			–			–		

(*Continued*)

1	2	3			4			5		
37	.000	+/−			+	100	−	++	100	−
	.001	−			−			−		
	to									
	.05	−			−			−		

TABLE IV—*Inoculum 10^6 cells, Y phase, no agar.*

25	.000	++	50	50	+++	100	−	++++	100	−
	.001	++	50	50	+++	80	20	+++	50	50
	.004	+	10	90	++	40	60	+++	50	50
	.007	+/−			++	20	80	+++	50	50
	.01	+/−			++	10	90	++	60	40
	.05	−			−			−		
30	.000	+	95	5	++	100	−	+++	100	−
	.001	+	40	60	++	95	5	+++	95	5
	.004	+	50	50	+	50	50	+++	95	5
	.007	+/−			+	50	50	+	80	20
	.01	−			−			+	80	20
	.05	−			−			−		
37	.000	++	90	10	++	90	10	+++	100	−
	.001	+++		100	++++	5	95	++++	10	90
	.004	−			−			−		
	to									
	.05	−			−			−		

TABLE V—*Inoculum 10^3 cells, M phase, no agar. (All M phase inocula were standardized nephelometrically against the counted number of Y phase cells.)*

25	.000	+/−			+	100	−	+	100	−
	.001	−			−			−		
	to									
	.05	−			−			−		
30	.000	−			−			−		
	to									
	.05	−			−			−		
37	.000	−			−			−		
	to									
	.05	−			−			−		

TABLE VI—*Inoculum 10^4 cells, M phase, no agar.*

25	.000	++	100	−	++	100	−	++	100	−
	.001	−			−			−		
	to									
	.05	−			−			−		

(*Continued*)

1	2	3			4			5		
30	.000	+	100	−	+	100	−	+	100	+
	.001	−			−			−		
	to									
	.05	−			−			−		
37	.000	+/−			+	100	−	+	100	+
	.001	−			−			−		
	to									
	.05	−			−			−		

TABLE VII—*Inoculum 10^5 cells, M phase, no agar.*

25	.000	+ +	100	−	+ +	100	−	+ +	100	+
	.001	+	100	−	+	100	−	+	100	+
	.004	−			−			−		
	to									
	.05	−			−			−		
30	.000	+	100	−	+	100	−	+	100	+
	.001	+	100	−	+	100	−	+	100	+
	.004	−			−			−		
	to									
	.05	−			−			−		
37	.000	+	100	−	+	100	−	+	100	+
	.001	−			−			−		
	to									
	.05				−			−		

TABLE VIII—*Inoculum 10^6 cells, M phase, no agar.*

25	.000	+ + +	100	−	+ + +	100	−	+ + +	100	+
	.001	+ + +	100	−	+ + +	100	−	+ + +	100	+
	.004	+ +	100	−	+ +	100	−	+ +	100	+
	.007	+	100	−	+ +	100	−	+ +	100	+
	.01	+	100	−	+	100	−	+	100	+
	.05	−								
30	.000	+ +	100	−	+ + +	100	−	+ + +	100	+
	.001	+ +	100	−	+ + +	100	−	+ + +	100	+
	.004	+	100	−	+ +	100	−	+ +	100	+
	.007	+	100	−	+	100	−	+ +	100	+
	.01	+/−			+	100	−	+	100	+
	.05	−								

ntinued)

	2	3			4			5		
	.000	+ +	100	–	+ +	100	–	+ +	100	–
	.001	+ +	20	80	+ + +	60	40	+ + +	60	40
	.004	+	10	90	+	50	50	+	50	50
	.007	+	5	95	+	50	50	+	50	50
	.01	–			–	–	–	–	–	–
	.05	–			–	–	–	–	–	–

TABLE IX—*Inoculum 2 × 10⁵ cells per ml medium, Y phase, no agar.*

5	.000	+ +	60	40	+ + +	90	10	+ + +	90	10
	.001	+ + +	tr[1]	100	+ + + +	5	95	+ + + +	5	95
	.004	+ + +	tr	100	+ + +	5	95	+ + + +	5	95
	.007	+ + +	tr	100	+ + +	5	95	+ + +	10	90
	.01	+ +	tr	100	+ +	5	95	c[2]		
	.05	+/–	–	–	+	tr	100	+ +	10	90
0	.000	+	90	10	+ +	90	10	+ + +	95	5
	.001	+	50	50	+ +	50	50	+ + +	50	50
	.004	+	50	50	+ +	50	50	+ + +	50	50
	.007	+	10	90	+	10	90	+ +	40	60
	.01	–	–		+/–	–		+ +	30	70
	.05	–	–		–	–		–		
7	.000	+ +	80	20	+ +	95	5	+ + +	95	5
	.001	+ + +	5	95	+ + +	5	95	+ + + +	5	95
	.004	+ + +	5	95	+ + +	5	95	+ + + +	5	95
	.007	+ +	5	95	+ + +	5	95	+ + + +	5	95
	.01	+	tr	100	+ + +	5	95	+ + +	tr	100
	.05	–			–			–		

TABLE X—*Inoculum 2 × 10⁶ cells, M phase, no agar.*

5	.000	+ +	100	–	+ + + +	100	–	+ + + +	100	–
	.001	+ +	100	–	+ + +	100	–	+ + +	100	–
	.004	+ +	100	–	+ + +	100	–	+ + +	100	–
	.007	+ +	100	–	+ +	100	–	c	–	
	.01	c			c			c		
	.05	–			–			–	–	
0	.000	+ +	100	–	+ + +	100	–	+ + +	100	–
	.001	+ +	100	–	+ + +	100	–	+ + +	100	–
	.004	+ +	100	–	+ +	100	–	+ +	100	–
	.007	+ +	100	–	+ +	100	–	+ +	100	–
	.01	+ +	100	–	+ +	100	–	+ +	100	–
	.05	–	–	–	+/–	–	–	+	100	–

[1] tr—trace.
[2] c—contaminated.

(*Continued*)

1	2	3			4			5		
37	.000	+ +	100		+ +	100	–	+ +	95	
	.001	+ +	40	60	+ + + +	70	30	+ + + +	50	5
	.004	+ +	50	50	+ + +	50	50	+ + + +	50	5
	.007	+ +	20	80	+ + +	10	90	+ + +	20	8
	.01	+	10	90	+	10	90	+ +	5	9
	.05	–	–	–	–	–	–	–	–	

TABLE XI—*Inoculum 2 × 10⁶ cells, Y phase, 0.04 per cent regular agar.*

25	.000	+ + +	100	tr	+ + + +	100	tr	+ + + +	100	tr
	.001	+ + +	20	80	+ + +	50	50	+ + + +	5	9
	.004	+ + +	10	90	+ + + +	20	80	+ + + +	5	9
	.007	+ + +	5	95	+ + + +	5	95	+ + + +	5	9
	.01	+ + +	5	95	+ + + +	5	95	+ + +	5	9
	.05	–	–		+/–	–		+ +	50	5
30	.000	+	90	10	+ + +	95	5	+ + +	100	tr
	.001	+	80	20	+ + +	90	10	+ + +	90	1
	.004	+	50	50	+ + +	70	30	+ + +	50	5
	.007	+	50	50	+ + +	50	50	+ + +	40	6
	.01	+	50	50	+ + +	20	80	+ + +	20	8
	.05	–	–		–	–		–	–	
37	.000	+ +	50	50	+ +	95	5	+ + +	95	
	.001	+ +	5	95	+ + +	10	90	+ + +	5	9
	.004	+ +	–	100	+ +	tr	100	+ + +	tr	10
	.007	+ +	–	100	+ + +	tr	100	+ + +	tr	10
	.01	+ +	–	100	+ + +	tr	100	+ + +	tr	10
	.05	–	–		–			–		

TABLE XII—*Inoculum 2 × 10⁶ cells, Y phase, 0.16 per cent regular agar.*

25	.000	+ + +	90	10	+ + +	95	5	+ + +	100	t
	.001	+ + +	10	90	+ + +	10	90	+ + +	50	5
	.004	+ +	–	100	+ + +	10	90	+ + +	50	5
	.007	+ +	–	100	+ +	–	100	+ + +	40	6
	.01	+ +	–	100	+ +	–	100	+ +	30	7
	.05	–	–		+	–	100	+ +	80	2
30	.000	+ +	95	5	+ + +	100	–	+ + +	100	
	.001	+	90	10	+ +	80	20	+ + +	95	
	.004	+	80	20	+ + +	60	40	+ + +	80	2
	.007	+/–	–		+ + +	50	50	+ + +	50	5
	.01	+/–	–		+	10	90	+ +	10	9
	.05	–			+/–			+ +	100	t

(*ntinued*)

	2	3			4			5		
7	.000	+	95	5	+ +	95	5	+ + +	95	5
	.001	+	5	95	+	5	95	+ +	20	80
	.004	+	–	100	+	tr	100	+ +	tr	100
	.007	+	–	100	+	tr	100	+ +	tr	100
	.01	–			+/–	–		+ +	tr	100
	.01	–			+/–	–		+ +	tr	100
	.05	–			–	–		–	–	–

TABLE XIII—*Inoculum 2 × 10⁶ cells, M phase, 0.04 per cent regular agar.*

5	.000	+ + +	100	–	+ + +	100	–	+ + +	100	–
	.001	+ +	100	–	+ + +	100	–	+ + +	100	–
	.004	+ + +	100	–	+ + +	100	–	+ + +	100	–
	.007	+ + +	100	–	+ + +	100	–	+ + +	100	–
	.01	+ +	100	–	+ +	100	–	+ + +	100	–
	.05	–			+/ ·			+	c	
0	.000	+ + +	100	–	+ + +	100	–	+ + +	100	–
	.001	+ + +	100	–	+ + +	100	–	+ + +	100	–
	.004	+ + +	100	–	+ + +	100	–	+ + +	100	–
	.007	+ + +	100	–	+ + +	100	–	+ + +	100	–
	.01	+ +	100	–	c			c		
	.05	–	–		–	–		–	–	–
7	.000	+/–	–		+	100	–	+	100	–
	.001	–			–			–		
	to									
	.05	–			–			–		

TABLE XIV—*Inoculum 2 × 10⁶ cells, M phase, 0.16 per cent regular agar.*

5	.000	+	100	–	+ +	100	–	+ + +	100	–
	.001	–	–	–	+	–	–	–	–	–
	.004	–	–	–	–	–	–	+	100	–
	.007	+	100	–	+	100	–	+	100	–
	.01	+/–	–	–	+	100	–	+	100	–
	.05	–	–	–	–			–		
0	.000	+	100	–	+ + +	100	–	+ + +	100	
	.001	+	100	–	+ +	100	–	+ +	100	
	.004	–	–	–	–	–	–	–	–	
	.007	+/–	–	–	+ +	100	–	+ +	100	
	.01	–	–	–	+/–	–	–	+ +	100	
	.05	–	–	–	–	–	–	–	–	
7	.000	+	100	–	+	100	–	+	100	–
	.001	–			–			–		
	to									
	.05	–			–			–		

(*Continued*)

1	2	3			4			5		

TABLE XV—*Inoculum 2 × 10⁶ cells per ml medium, Y phase, 0.04 per cent Noble agar.*

25	.000	+ + +	90	10	+ + +	95	5	+ + +	100	–
	.001	+ + +	10	90	+ + +	10	90	+ + +	10	90
	.004	+ + +	–	100	+ + +	tr	100	+ + +	10	90
	.007	+ + +	–	100	+ + +	tr	100	+ + +	10	90
	.01	+ + +	–	100	+ + +	tr	100	+ + +	5	95
	.05	–	–	100	+/–			+	10	90
30	.000	+ +	100	–	+ + +	100	–	+ + +	100	–
	.001	+ + +	90	10	+ + +	90	10	+ + +	95	5
	.004	+ +	50	50	+ +	50	50	+ + +	90	10
	.007	+ +	30	70	+ +	40	60	+ + +	50	50
	.01	+	10	90	+ + +	10	90	+ + +	50	50
	.05	–	–	–	–	–		–	–	–
37	.000	+ +	50	50	+ + +	c		c		
	.001	+ +	50	50	+ + +	20	80	+ + +	20	80
	.004	+ +	10	90	+ + +	10	90	+ + +	15	85
	.007	+ +	5	95	+ + +	10	90	+ + +	15	85
	.01	+ +	tr	100	+ +	5	95	+ + +	10	90
	.05				–			–		

TALLE XVI—*Inoculum 2 × 10⁶ cells, Y phase, 0.16 per cent Noble agar.*

25	.000	+ + +	100	–	+ + +	100	–	+ + +	100	—
	.001	+ +	5	95	+ + +	5	95	+ + +	50	50
	.004	+ +	5	95	+ +	5	95	+ +	30	70
	.007	+ +	5	95	+ +	tr	100	+ +	20	80
	.01	+ +	tr	100	+ +	tr	100	+ +	10	90
	.05	–	–		+/–			+ +	90	10
30	.000	+ +	95	5	+ +	100	tr	+ +	100	tr
	.001	+	50	50	+ +	70	30	+ + +	70	30
	.004	+	20	80	+ +	50	50	+ + +	50	50
	.007	+/–	–	–	+ +	30	70	+ +	30	70
	.01	+/–	–	–	+	+	80	+ +	10	90
	.05	–	–	–	+/–	–	–	+	50	50
37	.000	+	95	5	+ +	100	tr	+ + +	100	—
	.001	+ +	5	95	+ +	70	30	+ + +	50	50
	.004	+ +	–	100	+ +	50	50	+ + +	50	50
	.007	+	–	100	+ +	5	95	+ +	5	95
	.01	+	–	100	+	tr	100	+ +	tr	100
	.05	–			–			–		

(*continued*)

1	2	3			4			5		
	TABLE XVII—*Inoculum 2 × 10⁶ cells, M phase, 0.04 per cent Noble agar.*									
25	.000	+ +	100	–	+ +	100	–	+ +	100	–
	.001	+	100	–	+ +	100	–	+ +	100	–
	.004	+	100	–	+ +	100	–	+ +	100	–
	.007	+ +	100	–	+ +	100	–	+ +	100	–
	.01	+ / –			+ +	100	–	+ +	100	–
	.05	–			–			–		
30	.000	+ +	100	–	+ +	100	–	+ + +	100	–
	.001	+	100	–	+ +	100	–	+ + +	100	–
	.004	+	100	–	+ +	100	–	+ + +	100	–
	.007	+	100	–	+ +	100	–	+ + +	100	–
	.01	+ / –	–	–	+	100	–	+ +	100	–
	.05	–				–		–	–	
37	.000	–			–			–		
	to									
	.05	–			–			–		
	TABLE XVIII—*Inoculum 2 × 10⁶ cells, M phase, 0.016 per cent Noble agar.*									
25	.000	+	100	–	+ +	100	–	+ + +	100	–
	.001	+ / –	–	–	+	100	–	+	100	–
	.004	+	100	–	+ +	100	–	+ +	100	–
	.007	–	–	–	+	100	–	+ +	100	–
	.01	–			+	100	–	+ +	100	–
	.05	–			–			–		
30	.000	+	100	–	+ +	100	–	+ +	100	–
	.001	+	100	–	+ +	100	–	+ +	100	–
	.004	+	100	–	+ +	100	–	+ +	100	–
	.007	+	100	–	+ +	100	–	+ +	100	–
	.01	+ / –			+	100	–	+ + +	100	–
	.05	–			–	–		–	–	–
37	.000	+ / –			+	100	–	+	100	–
	.001	–			–			–		
	to									
	.05	–			–			–		
	TABLE XIX—*Inoculum 2 × 10⁶ cells, Y phase, 0.025 per cent silica jel.*									
25	.000	+ +	100	–	c			c		
	.001	+ + +	10	90	+ + +	50	50	+ + +	90	10
	.004	+ + +	10	90	+ + +	50	50	+ + +	60	40
	.007	+ +	5	95	+ + +	50	50	+ + +	60	40
	.01	+	5	95	+ +	50	50	+ +	60	40
	.05	–	–		+ / –			+	100	–

(*Continued*)

1	2	3			4			5		
30	.000	+	95	5	+ +	100	–	+ +	100	–
	.001	+	50	50	+ + +	50	50	+ + +	80	20
	.004	+	5	95	+ + +	50	50	+ + +	70	30
	.007	+	5	95	+ + +	40	60	+ + +	50	50
	.01	+/–			+ +	40	60	+ +	50	50
	.05	–			–			–		
37	.000	+	95	5	+ +	95	5	+ + +	100	–
	.001	+ +	50	50	+ + +	50	50	+ + +	5	95
	.004	+ +	5	95	+ + +	20	80	+ + +	tr	100
	.007	+ +	5	95	+ + +	10	90	+ +	tr	100
	.01	+ +	–	100	+ +	tr	100	+ +	tr	100
	.05	–			–			–		

TABLE XX—*Inoculum 2 × 10⁶ cells, M phase, 0.025 per cent silica jel.*

25	.000	+ +	100	–	c			c		
	.001	+ +	100	–	+ +	100	–	+ + + +	100	–
	.004	–	–		–			–	—	–
	.007	–	–		+/–			+	100	–
	.01	–	–		+/–			+	100	–
	.05	–	–		–			–		
30	.000	+/–			+	100	–	+ +	100	–
	.001	+	100		+	100	–	+ +	100	–
	.004	–	–		+	100	–	+	100	–
	.007	–	–		+/–			+	100	–
	.01	–	–		+/–			+	100	–
	.05	–	–		+/–			–		
37	.000 to .05	–			–			–		

that after 7 days the cultures containing no cysteine still show a significant percentage of Y phase cells at all incubation temperatures, it being inexplicably high at 25°C, but that after 14 days incubation only the culture incubated at 37°C still contains any amount of Y cells; even these disappear entirely after 21 days' incubation.

Using an M phase inoculum comparable to 10^6 Y cells/ml of culture medium (Table VIII) it was not possible to effect M→Y conversion except at 37°C. Under these conditions and with 0.007 *M* cysteine, Y phase growth as high as 95 per cent was found after 7 days' incubation; subsequent incubation markedly decreased this amount of Y phase growth.

When the inoculum of Y phase cells was increased to 2×10^6 cells per ml medium there was better growth at all temperatures of incubation and nearly 100 per cent preservation of the Y phase at 25°C (compare Tables VII and IX). Note here too (Table IX) that 30°C is less effective in maintaining the Y phase growth than 25°C. An inoculum of the M phase comparable to 2×10^6 cells per ml of medium (Table X) again permitted M→Y conversion only at 37°C.

The addition of 0.04 per cent regular agar (Difco) to the culture made no alterations in the proportion of Y to M phase growth when the Y phase inoculum was used (compare tables IX and XI) except that overall growth was slightly better at all incubation temperatures, and Y phase growth was little less in the cultures incubated at 25°C.

When 0.04 per cent regular agar was used with an M phase inoculum (Table XIII) it is most significant that growth at 37°C was nearly completely suppressed, only (+) growth taking place in the culture containing no cysteine. Again note that only M phase growth was present at 25° and 30°C incubation. Increasing the agar concentration to 0.16 per cent reduced the growth of the Y phase inoculum slightly (compare Tables XI and XII). However, increasing the agar concentration to 0.16 per cent when using the M phase inoculum (Table XIV) reduced overall growth markedly at all temperatures and again permitted no growth at 37°C at any concentration of cysteine.

This suppression of growth of the M phase inoculum when agar was added occurred whether regular agar (Tables XIII and XIV), Difco's washed agar (Noble agar) at the same concentrations (Tables XVII and XVIII) or washed silica jel in a concentration of 0.025 per cent (Table XX) was used. The 0.04 per cent Noble agar (Table XVII) and silica jel (Table XX) were so effective that no growth at all was found at an incubation of 37°C. The silica jel reduced markedly the growth at 25° and 30°C when compared to the mate cultures containing no agar or any of those containing regular or washed agar.

DISCUSSION

Since no yeast phase growth was found with an inoculum of 10^5 cells per ml of culture medium or less, regardless of the incubation time or temperature, cysteine concentration, phase of inoculum, or varying combinations of these conditions, it appears clear that a minimum mass of inoculum is essential for the induction or maintenance of the yeast phase. Under conditions where less than such a critical mass of inoculum is used only the

mycelial phase can multiply. If the cultural conditions of environmental temperature and cysteine concentration are such as to restrict mycelial growth, and the mass of inoculum is less than that required for yeast phase growth, one should expect sterility if both sets of conditions are simultaneously imposed on a culture. This premise is borne out by the data here reported, since no growth occurred at the incubation temperature of 37°C at any of the cysteine concentrations when the inoculum of either phase was 10^5 cells or less (Tables I–III, V–VII).

Pine [2], using a different synthetic medium, also reported the necessity of a minimum mass of cells to initiate Y phase growth. Although Pine used strains of *H. capsulatum* and a medium differing from that used here, it is interesting that his finding of a number of cells greater than 5×10^4 per ml of medium as essential to initiate Y phase growth is in accord with the data here reported.

It will also be noted that the amount of mycelial growth found was (aside from the size of inoculum) directly proportional to the decrease in incubation temperature and inversely proportional to the increase in molar concentration of cysteine.

As might be expected, 37°C is, in general, a more effective temperature for the maintenance of the Y phase inoculum than 25° or 30°C, the latter being the least effective of the three. However, in a few instances little difference was evident at 25° or 37°C (note Tables IX and XV). It would thus appear that the concentration of –SH groups (or cysteine) is more critical for the maintenance of the Y phase than 37°C. The fact that a molecule containing reduced sulfur is not a mandatory requirement for yeast phase growth of *H. capsulatum* was shown in the precise studies of Salvin [5]. He found that 10^{-4} *M* l-cystine maintained Y phase growth, whereas 5 times this concentration of 1 (+) cysteine was necessary to do so. The reasons offered were that cystine contained twice as much sulfur as cysteine, mole for mole, and that the molecule was of a nature to be utilized by the organism, since such compounds as serine, cysteic acid, or glutathione were not capable of maintaining pure yeast phase growth under comparable conditions. We are thus led to believe that a molecule containing a sulfur group is necessary for Y phase growth of *H. capsulatum*, the organism usually requiring the intact –SH group because of its limited capacity to reduce sulfur-containing molecules. The role of 37°C appears to be the maintenance of optimum conditions for the activity of certain enzymes which direct the M→Y transition.

There is no reason to believe that Y phase growth, once having been initi-

ed, could not be propagated indefinitely at temperatures less than 37°C if e –SH content or perhaps O–R potential (see later discussion) of the edium were maintained at some optimum level. The fact that M→Y con-rsion has not been accomplished at an incubation temperature less than 7°C[1] indicates that the metabolic pathway (*s*) governing the maintenance of phase growth and those directing M→Y transformation are distinct from ach other.

The addition of agar, washed or regular, or silica jel to the medium ould serve to reduce the oxidation-reduction potential of the SH-containing ıedium still further. One should expect that Y phase maintenance would be ıus improved unless growth without the agar or silica jel was sufficiently lose to optimum already. The introduction of additional factors favoring Y hase growth would also mean the presence of conditions which restrict M hase growth. A combination of such factors used here, i.e., cysteine, 37°C, nd the agar or silica jel, did not permit M→Y conversion because the M hase inoculum was prevented from growing at all. Removal of the agar or ilica jel permitted M→Y conversion at 37°C when cysteine was present. emoval of the cysteine from the combination which prevented growth of he M phase inoculum permitted only meager M phase growth and in two nstances (Tables XVII and XX) did not alter the course of events at all. It hus appears that the presence of agar or silica jel at the low concentrations ısed here, and without the presence of cysteine, reduces the O–R potential ufficiently to suppress mycelial growth.

A puzzling feature is the good mycelial growth at 37°C in the presence of gar or silica jel when the inoculum was originally in the yeast phase, the mount of mycelial growth being as great as in comparable cultures without he agar (compare Tables IX, XI, and XIX). In other words, a mycelial hase inoculum will not grow at 37°C in the presence of 0.025 per cent silica el but will grow as a mycelium if the inoculum was initially in the Y phase.

The explanation for this might be forthcoming from some preliminary tudies done in this laboratory measuring O–R potentials during the course f growth of cultures under conditions comparable to those described here xcept that no agar or silica jel was used. It is our intent to describe these tudies in detail at another time, but the following facts are most pertinent for ıis discussion. In those cultures with (+ + +) or (+ + + +) growth, and

[1] In a few instances cultures containing M phase inocula and cysteine in various concentrations ıowed Y phase growth at 25°C. In every case it was found that these cultures had become con-ıminated with bacteria. That such contamination may supply substances essential to M→Y imorphism not available to mycelium growing at temperatures less than 37°C is presently being ıvestigated.

over 4 days old with 85 per cent or more of the total growth in one phase, it was found that, regardless of the phase of the initial inoculum and conditions of culture, the mean oxidation-reduction potential was +129 mv ± 4 mv for the yeast phase and +89 mv ± 13 mv for the mycelial phase cultures. These differences are statistically significant ($P = 0.001$). This would indicate that Y phase growth, once having been initiated by the conditions of the culture, would raise the O–R potential sufficiently to permit Y→M transformation in the absence of concentrations of cysteine necessary to maintain the Y phase. At very low O–R potentials an M phase inoculum cannot convert to the Y phase because it cannot grow. The role of the cysteine in maintaining Y phase growth or in permitting M→Y dimorphism at 37°C must be one of lowering the O–R potential of the medium, since it is doubtful if any unoxidized –SH groups remain in the medium after a day or two. However, since Y phase growth results in a high O–R potential compared to M phase growth the conclusion of Pine [2] that "... the requirement for the –SH group is to maintain the viability of the organism until growth commences" probably correctly describes the course of events governing dimorphism, except that it directs attention more to the role of cysteine in supplying –SH groups than to its effect on the O–R potential of the medium.

It is logical to propose that once the O–R potential of the medium (among other conditions) has determined the phase of the organism, the metabolic rate of the organism would determine the O–R potential of the medium. If the Y phase were metabolically more active than the mycelial phase, it might reach equilibrium at a higher O–R potential than the less active mycelial phase. Nickerson and Edwards [1] reported that, at least in *Blastomyces dermatitidis* and *B. brasiliensis*, the Y form consumes approximately five times as much oxygen on a dry weight basis as the M form.

A more refined series of measurements better correlated with phase growth might be made in a chemostat where a relatively constant O–R potential of the culture medium could be maintained. It is also possible that the presence or absence of cysteine may direct phase changes independent of the O–R potential of the medium. These and related problems are now under investigation.

SUMMARY

Employing a synthetic medium to which were added varying concentrations of cysteine and incubation temperatures of 25°, 30°, and 37°C, Y phase cells of *H. capsulatum* were maintained preponderantly in this phase for 3 weeks at 25°C with as little as 0.001 *M* cysteine. Such maintenance of the Y

ase required an inoculum of at least 2×10^6 cells per ml of medium. ocula of 10^5 cells or less permitted no Y phase growth at any temperature of ubation used and at any concentration of cysteine from 0.001 to 0.05 *M.* ne addition of 0.04 per cent agar, washed or regular, or 0.025 per cent ica jel to the medium containing cysteine at any concentration completely hibited growth of the mycelial phase inoculum at 37°C. Since these same nditions permitted good Y phase growth, it was concluded that the condi- ns of culture and hence the biochemical pathways directing M→Y di- orphism and those necessary to maintain Y phase growth are independent each other. The ramifications of these findings are discussed.

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SPECIES DIFFERENCES IN THE BRUSH BORDERS OF KIDNEY AND INTESTINE AS REVEALED BY REFRACTOMETRY WITH THE PHASE MICROSCOPE

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It has recently been shown that details of unstained sections of fixed tissue may be investigated by a refractometric technique [4, 5, 3, 8]. This entails study of the phase-contrast behaviour of unstained sections mounted in media of known refractive index (R.I.). If the R.I. of a particular constituent of the section exceeds that of the mountant it will appear darker than the background or phase positive; if less, it will appear brighter—phase-negative. The degree of contrast between object and background is proportionate to the difference between their respective R.I. If the difference is large, the contrast will be high and, if slight, low. If the refractive indices coincide contrast will be zero and the object more or less invisible.

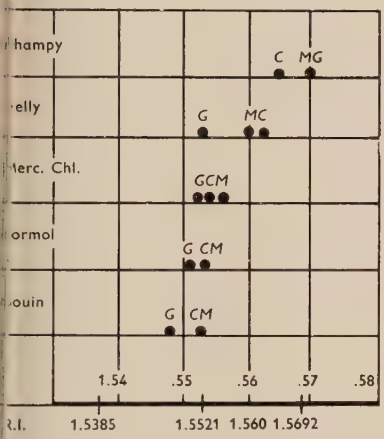
Previous work [7, 8] with this technique indicated that certain structures apparently indistinguishable from each other by orthodox staining methods might nevertheless be separable on the basis of their R.I. This possibility has been investigated further. The striated borders of intestinal and renal epithelial cells belonging to different species cannot be distinguished from one another by their staining reactions and the renal peritubular basement membranes all look alike. In the present experiments the R.I. of these structures has been investigated and, as will be described below, small but definite species variations were brought to light.

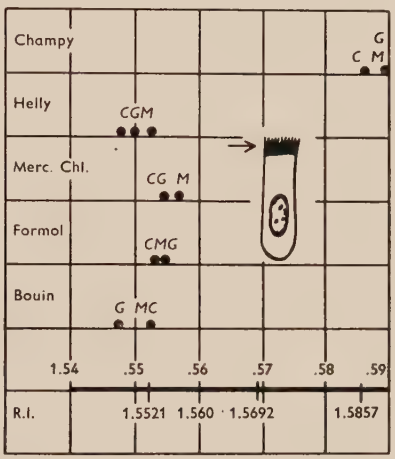
MATERIAL AND METHODS

The kidney and jejunum of normal cat, mouse and guinea pig were used. Samples of each organ from these species were fixed, dehydrated and cleared together in the same vessel and were blocked together in wax, so that they could be cut and mounted at the same time ensuring that the thickness of the section was identical. The following fixatives were employed: formol, mercuric chloride and formol, Bouin, Helly and Champy fluids. The microscopic technique was as before [8]; after deparaffinizing sections were transferred to dest. water [13], then dehydrated. A chosen field was examined and photographed by phase-contrast in a series of mountants of known R.I. The light transmission of the brush border and peritubular basement membrane were compared to that of the background (light transmitted through mountant alone).

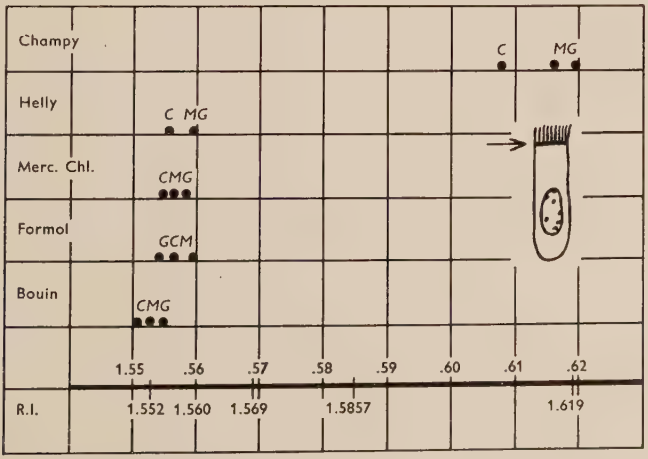
RESULTS

The brush borders were easily seen in the unstained sections (Figs. 1, 7) d two layers distinguished. The upper of these comprised the actual striae, hilst the lower appeared as a continuous line.



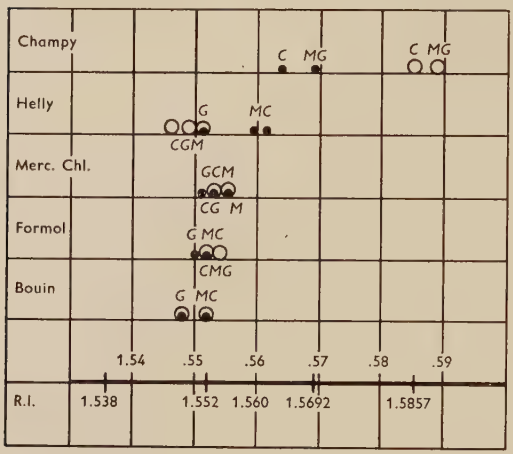


ext-fig. 1 (*left*). Species variations in the refractive index of the kidney brush border, fixed ith 5 different solutions. *C*, cat; *M*, mouse; *G*, guinea pig.
ext-fig. 2 (*right*). Species variations in the R.I. of the intestinal brush border (upper area own by arrow), fixed with 5 different solutions.

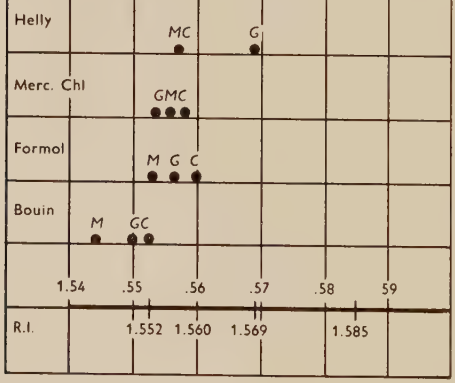


ext-fig. 3. Species variations in the R.I. of the intestinal brush border (lower area, shown by row), fixed with 5 different solutions.

The upper area appeared phase-positive against a lighter background i mountants of low R.I. (1.5243) and phase-negative when the mountai R.I. was high (1.58 or more). The most useful results were obtained wit



Text-fig. 4. Comparison of results shown Tables I and II. ●, Kidney brush borde ○, intestinal brush border (upper).



Text-fig. 5. Species variations in the R.I. of th renal peritubular basement membrane, fixe with 5 different solutions.

mountants whose R.I. lay close to that of the border. Under these circum stances there were small but definite species variations in the sense and degre of contrast (Figs. 2–3, 4–6, 7–9). The results of the observations are summar ized graphically in Text-figs. 1 and 2. As these show, the R.I. of the borde was influenced by the fixative. The osmic acid was probably responsible fo the very high value obtained for tissue fixed in Champy's fluid. Othe fixatives, e.g. formol, Bouin's fluid, gave a much lower value, whilst Helly

id had an intermediate effect. This finding was checked by blocking and ting together samples from the same organ which had been fixed in various utions. When sections were examined in bromo-benzene (R.I. = 1.5600), le differences in the light transmission of the samples were revealed. This nfirmed previous results [8].

Similar species variations were encountered with the lower area of the lush border (Text-fig. 3). This continuous basal line [12, 15] was more tinct in the intestinal than in the renal epithelial cells (Figs. 7, 8). The R.I. the renal and intestinal border seemed to differ in the same animal (Text-. 4).

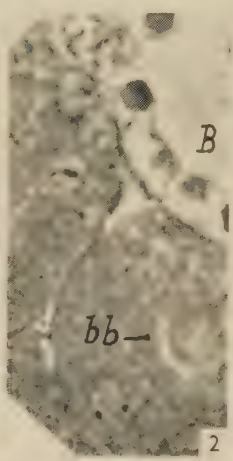
Species variations were also brought to light in the R.I. of the basement embrane around the renal convoluted tubules (Text-fig. 5).

The effect of different fixatives upon the R.I. of the structures studied varied ite noticeably with species; for instance, as shown in Text-fig. 2, after Bouin ation the species order of increasing R.I. was guinea pig, mouse cat, whereas ter Helly it was cat, guinea pig, mouse. This evidently reflected underlying nstitutional differences in the different species.

DISCUSSION

The measurement of R.I. of tissue sections by the method used is of course bject to certain technical pitfalls. First there are the difficulties inherent in hase-contrast microscopy, particularly the possibility of being misled by halo" effects [14]. These however could easily be discerned and did not give se to difficulty in interpretation. Again, since phase change is a product the thickness as well as the R.I. of an object [2, 6], it would be pointless make comparative observations on sections, unless their thickness was a onstant. This was achieved by blocking the tissue samples close together in ax, so that the experimental sections were cut at the same microtome roke. Second, there is the possibility of subjective error in assigning contrast alues. This seems unlikely (Figs. 2–3, 4–6, 7–9). Third, it has to be borne in ind that the absolute value of R.I. may be influenced in several ways. s described above, the fixative plays an important role; and washing sec-ons in water can also affect the value [7, 8]. This latter procedure has, of ourse, been shown on other grounds to alter the appearance [13, 10] and e histochemical attributes of sections [9].

There are of course certain obvious species differences in homologous imal cells. For instance, there are wide differences in the sizes of some lls, such as erythrocytes. Also, there are regional and functional differences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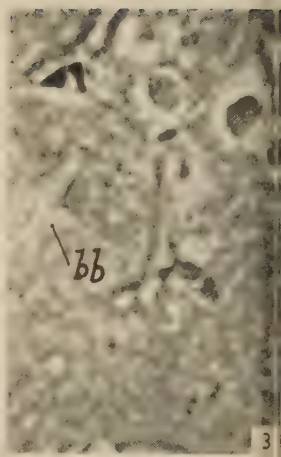
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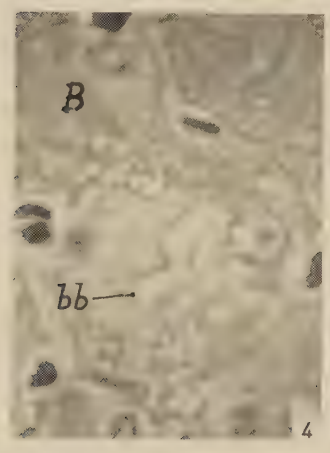
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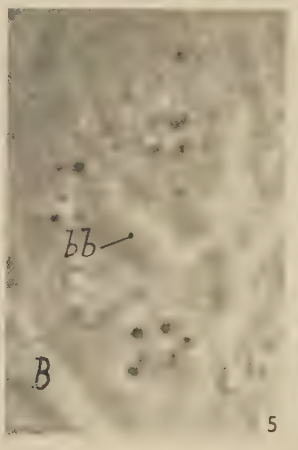
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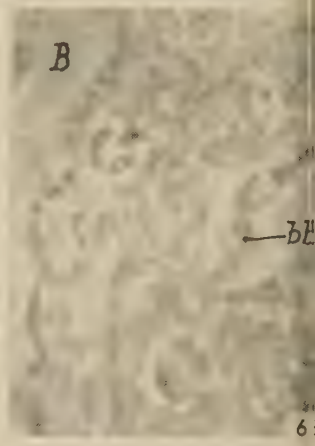
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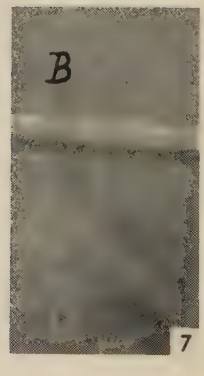
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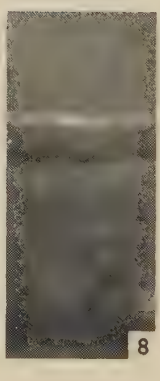

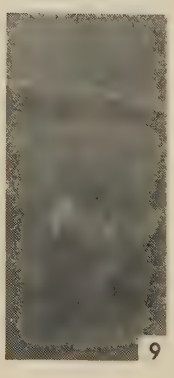

hich may affect the appearance of a cell [11]. Some structures, however, e cell membranes or brush borders have the same appearance in different ecies when stained by routine or even special methods. The results described ove indicate that they nevertheless possess unequal indices of refraction. his must reflect some underlying constitutional differences, which, perhaps, e related to its important functional role.

The extent of species differences, as described above, was to some extent unction of fixative. With Champy's fluid, contrast was high and with formol uch less so. It seems very likely that in the first case osmium or chromium mbined with a substance in the border to produce an additive compound. his is in agreement with the findings of Bahr [1] and Porter and Kallman [17] no used, however, different material and technique. Thus the species differ-ces observed after Champy fixation might have reflected variation in the nount of substances having an affinity for osmium or chromium. With rmol or Bouin, on the other hand, it is possible that water soluble sub-ances such as mineral salts [16, 18] and carbohydrates leached out into lution in the fixative. There might then be left behind only the water-in-luble supporting framework of the border, and this might minimize species fferences.

SUMMARY

1. The refractive indices of the brush border of intestinal and kidney ithelium and the basement membrane of kidney tubuli were investigated.

2. Species differences in these structures could be seen when they were amined by phase-contrast microscopy in mountants of known R.I.

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ll photomicrographs were of unstained sections in phase illumination, × 95 oil immersion jective.

ig. 1. Kidney convoluted tubulus differentiated optically, mounted in chloro-benzene (R.I. = 5243).

igs. 2–3. Species differences in refractive index of kidney epithelium fixed in Champy's fluid. rush border of proximal convoluted tubuli (pars recta) of mouse and cat. Sections mounted in romo-benzene (R.I. = 1.5600). *Fig. 2.* Brush border of mouse appears phase-positive in com-arison with the background (*B*). Brush border (*bb*). *Fig. 3.* Brush border of cat is phase-neutral weak negative.

igs. 4–6. Species differences in R.I. of kidney epithelium. Brush border (*bb*) of proximal convo-ted tubuli (pars convoluta) of mouse, guinea pig and cat, fixed in Helly's fluid. Sections mounted nitro-benzene (R.I. = 1.5521). *Fig. 4.* Brush border of mouse is phase-neutral. *Fig. 5.* Brush rder of guinea pig appears weak phase-positive. *Fig. 6.* Brush border of cat is phase-positive.

igs. 7–9. Species differences in R.I. of intestinal brush border (jejunum) of cat, guinea pig and ouse, fixed in Helly's fluid. Sections mounted in nitro-benzene (R.I. = 1.5521). *Fig. 7.* Brush rder of cat is phase-negative. *Fig. 8.* Brush border of guinea pig is phase-positive. *Fig. 9.* rush border of mouse is phase-neutral or weak positive. The brush border is covered by a thin yer of mucus, which is strong phase-negative.

3. The refractive index of fixed tissues of different animals varied accord ing to the fixative employed.

I am greatly indebted to Dr. N. M. Hancox for his kind help in preparing th manuscript.

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ME CYTOLOGICAL OBSERVATIONS ON THE EFFECT OF RIBONUCLEASE ON *COCCIDIOIDES IMMITIS*[1,2]

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BONUCLEASE has been shown to penetrate several types of living cells and oduce effects which, in some cases, are reversible. Onion root-tips treated th the enzyme show a period of increased synthesis of ribonucleic acid llowed by a diminution in concentration of this compound [2], while in noebae, the loss of ribonucleic acid is immediate though reversible in a rtain per cent of the organisms [3]. Abnormalities in mitosis were induced onion root tip cells by ribonuclease [7]. Influenza virus replication in chick orioallantoic membrane cultures was inhibited by the addition of ribo-clease to the culture medium [9]. The inhibition of bacteriophage replica-n within bacterial protoplasts has been reported by Brenner [4] who monstrated that the effect is due to lysis of the protoplasts. He believes at ribonuclease cannot penetrate the protoplasts.

The failure of ribonuclease to penetrate the intact walls of living bacteria as reported by Brenner [4] and Beljanski [1]. Reports are not in agreement some of the effects of ribonuclease on fixed bacteria. Minck and Minck 1] found that metachromatic granules in *Corynebacterium diphtheriae* are sceptible to the action of ribonuclease, while Wachstein and Pisano [14] ported that the enzyme had no effect on metachromatic staining of the same ganism.

Although ribonuclease treatment of fixed bacteria has been found useful r demonstrating nuclei [13], Lamanna and Mallette [10] have stated that e technique is not applicable to living bacteria.

In the present study the effect of ribonuclease on the staining of nuclei, itochondria, and metachromatic granules in living cells of *Coccidioides mmitis* has been investigated. Evidence will be presented which suggests that e enzyme penetrates the intact cell wall and that some of its effects on the ganism are reversible.

[1] This investigation was supported in part by the State of Washington Fund for Medical and ological Research.

[2] Portion of a dissertation presented by the senior author as partial fulfilment of the re-irements for the degree of Doctor of Philosophy at the University of Washington, Seattle, ashington.

MATERIALS AND METHODS

The organism used in the present experiments, obtained from a human case o coccidioidomycosis in 1952, was maintained by transfer in trypticase-soya-yeas extract broth. The morphology of the organism in culture and in infected animals i typical of *C. immitis*.

All tests were made with organisms grown in a medium of the following composition NH_4Cl, 1 per cent; Na_2HPO_4, 0.4 per cent; KH_2PO_4, 0.5 per cent; $MgCl_2$, 0.01 per cen glucose, 2 per cent. Cultures were incubated at 37°C. For the production of sporangi the medium was inoculated with a suspension of organisms from infected mous brain and incubation was carried out in an atmosphere of nitrogen containing 1 per cent O_2 and 10 per cent CO_2.

Treatment with crystalline ribonuclease[1] was carried out at 37°C for varying period of time using a concentration of 0.1 per cent at pH 6.0. Following treatment wit ribonuclease or acid, the nuclei were stained with Giemsa stain for 20 to 24 hours a room temperature; dehydrated in 3:1 butyl:ethyl alcohol, cleared in xylol, an mounted in resin.

Three different procedures were used for supravital staining of mitochondri Janus green B in 10^{-4} dilution in distilled water, neotetrazolium in 10^{-3} dilution i distilled water, and the nadi reagent. The organisms were mounted in the reagents o a slide and the coverslip sealed with vaspar.

Metachromasia was demonstrated with 0.01 per cent azure A in 30 per cent alcoh according to the method of Kramer and Windrum [8] and mounted in glycerine jell

Following ribonuclease treatment the organisms were washed twice with distill water before staining. Unless otherwise stated, all tests were performed on living cell

RESULTS

Nuclei.—Stained nuclei are well defined in cells of 3- and 23-day culture submitted to ribonuclease treatment for 24 hours. In the cells of youn hyphae some basophilic material still remains in the cytoplasm, but nucl are stained more intensely than the cytoplasm. Nuclei could not be demon strated by Giemsa staining in either fixed or unfixed cells without prior trea ment to remove cytoplasmic basophily. In 23-day cultures many of the old cells appear to be autolyzed. No nuclei could be demonstrated in these cell

The nuclei were found to occur in 4 distinct configurations: groups granules, discs, rings, and crescents. These forms probably represent differe stages in the nuclear cycle. At the stage which may correspond to metaphas 3 or 4 granules are present in the nucleus. These may represent the chrom somes. However, owing to the disparity between chromosome counts an genetic analyses of linkage groups reported in other cells with chromoson

[1] Nutritional Biochemicals Corporation, Cleveland, Ohio.

e near the limit of resolution of the microscope [5, 6], it did not appear visable to attempt a chromosome count of *C. immitis* whose average nuclear ameter is 1.0 μ.

Nuclei were seen to pull apart during division, but no achromatic figure was monstrated by the staining methods used. Thus, while it appears probable at nuclear division takes place by mitosis, further studies will be required establish this point. In narrow hyphae, 1.5 μ or less, the plane of division parallel to the long axis of the cell. Deviation from this position becomes eater with increasing width of the hyphae until in hyphae more than 3 μ diameter, division is generally perpendicular to the long axis of the cell. Cells in young hyphae are frequently multinucleate, as are the majority the arthrospores. In chlamydospores and developing sporangia the nuclei e distributed at the periphery of the cell, close to the wall. Septum formaon [12] is not synchronized with nuclear division. The older cells of the yphae, 30 μ or more from the tip, are uninucleate and the internuclear istances are increased.

Mitochondria.—Supravital staining of mitochondria with Janus green B and eotetrazolium is diminished in intensity in cells treated with ribonuclease r 6 hours. The reaction with the nadi reagent, however, is not altered. reatment with ribonuclease for 24 hours causes a delay in the appearance f the reaction with neotetrazolium and Janus green B. After 36 hours of nzyme treatment the nadi reaction is also affected. The staining reactions ppear to be abolished at this time. However, after standing for 48 hours ie mounts of the organisms begin to show the characteristic reactions for ach reagent with a distribution of stained granules similar to that of control ells without ribonuclease treatment. After standing for 48 hours the neotetazolium reaction of both control and enzyme treated organisms show pinktained lipid granules as well as blue-stained mitochondria.

Metachromatic granules.—The distribution of metachromatic material vaies with the age of the cell. In young cultures, 5 days or less, cells are found vhich show considerable diffuse metachromasia. Irregular accumulations f intensely stained metachromatic material and small deeply-stained granles are also seen scattered throughout the hyphae. Young arthrospores are lled with metachromatic material.

Azure A staining of 2-week cultures shows many metachromatic granules, ut an absence of diffuse staining. Although some of the granules are in the orm of rings similar to nuclei, their distribution is unlike that of nuclei. hlamydospores are filled with metachromatic material and arthrospores ontain large clumps of the material. Metachromatic material in sporangia

is distributed either in plaques along the wall, in granules of varying sizes, or in some cases, diffusely.

Treatment of 3-, 7-, 16-, and 37-day cultures with ribonuclease for 24 hours prior to staining with azure A resulted in nearly complete loss of metachromasia in 3-day cultures, considerable diminution of metachromasia in 7-day cultures and some reduction of metachromasia in older cultures.

Control cultures of the same ages were dehydrated in 3:1 butyl:ethyl alcohol for 5 minutes after staining, then cleared and mounted in resin. Organisms thus treated show a considerable loss of metachromasia in cells from 3- and 7-day cultures while most of the metachromatic material in 16- and 37-day cultures is retained.

Similarly, control cultures treated with distilled water at 80°C for 10 minutes prior to staining with azure A show some retention of metachromasia in 16- and 37-day cultures and loss of material in the younger cultures.

Viability of organisms after ribonuclease treatment.—After 24- and 48-hour ribonuclease treatment, both young (5-day) and old (25-day) cultures were shown to be viable after inoculation into the test medium. In Giemsa stains of these subcultures the nuclei are again completely obscured by the basophily of the cytoplasm. After 36 hours in culture, no areas remain where nuclei are visible by this staining method. All cells of the younger cultures appear to be viable. However, in older cultures many more cells appear dead in subcultures of ribonuclease treated organisms than in control cultures of the same age.

DISCUSSION

The effect of ribonuclease in removing cytoplasmic basophily to the extent that nuclei can be visualized with basic dyes tends to show that the enzyme can penetrate the walls of the living fungus. The fact that organisms submitted to such enzyme treatment have been shown to be viable after the treatment and that cytoplasmic basophily can be regained in culture so that nuclei again are obscured, suggests that the penetration of the enzyme is not dependent upon death of the cell during the prolonged incubation period. It seems possible that more prolonged ribonuclease treatment of living, intact bacteria may result in penetration of the enzyme.

The effect of ribonuclease on the staining of mitochondria tends to substantiate the view that the enzyme penetrates the living cell wall. The variation in staining of mitochondria by the three methods used suggests that the enzymes mediating the neotetrazolium reduction are more sensitive to the level of ribonucleic acid. The prolonged delay in the appearance of stained

itochondria after 36-hour ribonuclease digestion may indicate the period uring which the results of the enzyme digestion are reversed, a period of synthesis, or indicate the extent to which the system is disrupted by removal ribonucleic acid as shown by the decrease in the rate of the reactions ediated by the mitochondrial enzymes.

Ribonuclease, like hot water and alcohol, varies in its effect on metachroatic staining with the age of the organism. Young cells are more susceptible loss of metachromatic material by each of these methods. This effect might e due to the difference in cell wall thickness, since the cell walls of older yphae are considerably thicker than those of young cells. However, the alls of some arthrospores of 7-day cultures are as thick as those of vegetative yphae of 37-day cultures. These spores yielded their metachromatic material readily to ribonuclease, hot water, and alcohol as did the vegetative cells 7-day cultures. This suggests that the change with aging is in the composition arrangement of the molecule of the material staining metachromatically. hus the discrepancies in the results of ribonuclease treatment on the staining f metachromatic granules of *Corynebacterium diphtheriae* [11, 14] might be ccounted for in part by a difference in the age of the organisms, although ne test conditions employed were not identical in the two cases.

SUMMARY

The effects of ribonuclease digestion of living cells of *Coccidioides immitis* n staining of nuclei, mitochondria, and metachromatic material is described. vidence is presented suggesting that the enzyme penetrates the wall of the ving cell since some of the results of enzyme action are reversible and the ells of young cultures are viable after prolonged enzyme treatment.

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THE QUANTITATIVE CYTOCHEMICAL ESTIMATION OF ARGININE

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More methods are urgently required for measuring the various chemical components of the nucleus in the same way that desoxyribose nucleic acid (DNA) can now be measured by means of the Feulgen reaction. The results described in this note may provide the basis for the quantitative cytochemical determination of arginine.

In the Sakaguchi reaction a red or orange-red colour is produced when a solution of arginine, or protein containing arginine, is treated with either α-naphthol [6] or 8-hydroxyquinoline [7] and sodium hypochlorite in the presence of an alkali. The original method has been modified in various ways for the colorimetric estimation of arginine in solution [4, 5] and has also been adapted to histochemical use [1, 8, 9]. The reaction, although specific [1] has always been difficult to control and gives a weak, unstable colouration. For these reasons it has not been suitable for application to quantitative cytochemistry.

A new modification, based on Baker's method, has now been devised however, in which 2,4-dichloro-α-naphthol [2] is used instead of a α-naphthol. This compound reacts rapidly to give an intense red colour which, by mounting the section or smear in a suitably alkaline medium, can be stabilised for a sufficient period to allow reliable photometric measurements to be made. Its validity as a quantitative method has been tested by measuring the total light absorption of the red coloured complex in individual calf thymus nuclei and bull sperm by means of an integrating microdensitometer [3] and comparing the ratio of these amounts with a ratio obtained, by biochemical procedures, by Vendrely, Knobloch and Vendrely [10]. Using a colorimetric modification of the Sakaguchi test, these authors have estimated that individual calf thymus nuclei and bull sperm contain respectively 1.49×10^{-6} and 2.16×10^{-6} γ of arginine. It was proposed, therefore, to test the new method by comparing the ratio of these amounts (1.45) with our ratio of the mean absorption values.

EXPERIMENTS

In the first experiment approximately 0.5 g of fresh calf thymus was ground by nd and a clean suspension of nuclei obtained after three centrifugations in ice-cold rmal Tyrode solution. The living bull sperm were treated in a similar way by ing washed and centrifuged three times in ice-cold Tyrode. Smears were made mediately after the final centrifugation and fixed for 15 min. in Lewitsky's ative (equal volumes of 10 per cent formalin and 1 per cent chromic acid). They re then washed for 5 min. in each of three successive changes of distilled water. order to prevent loss of material during the reaction the smears were coated with lloidin [1] by being passed through an alcohol series, dipped in 1 per cent celloidin equal parts of ether and absolute ethanol and air-dried.

Since the main object of the experiment was to compare the arginine content of e thymus nuclei and sperm, care was taken to ensure that they each received exactly e same treatment throughout. Thus smears of each were taken in pairs through ation, washing and celloidin coating and were kept together in the reaction mix- re. This consisted of: 30 ml 1 per cent NaOH; 0.6 ml 1 per cent 2,4-dichloro-α- phthol in 70 per cent ethanol; 1.2 ml of the proprietary product "Milton" which as been found to be suitable as a stable source of 1 per cent sodium hypochlorite [1]. These constituents were mixed immediately before use and the smears, having en taken quickly through an alcohol series to water, were placed in the mixture r 6 min. (the colour appeared after 1–2 min.). They were then rinsed rapidly in per cent urea, placed in 1 per cent NaOH for 5 min. and finally mounted in a ixture of 9 volumes glycerol to 1 volume 10 per cent NaOH. The red colour is re- ined only in a strongly alkaline medium; in neutral media it fades to a dull orange- d and turns yellow in acid. Measurements of the amount of colour were made by etermining the total absorption at 546 mμ, a mercury arc lamp and Wratten 77A nd 58 filters being used.

RESULTS

In the first experiment (Fig. 1 *a*) the ratio of the mean values obtained was .82. A repeat experiment with different samples of thymus and sperm gave ratio of 1.81 (Fig. 1 *d*). In both experiments comparisons with fresh smears f unwashed sperm showed that in the sperm, at least, Tyrode-washing did ot lead to a significant change in total absorption.

A possible explanation for the discrepancy between our ratios and that f 1.45 obtained by Vendrely *et al.* was thought to lie in the fact that these uthors employed *M*/18 citric acid as a medium for isolating the thymus uclei. A further experiment was therefore made in which the nuclei were solated and the sperm washed in citric acid of this molarity. The results lotted in Fig. 2 *a* show a drop in the ratio of means to 1.61. A repetition f this experiment (Fig. 2 *b*) gave a ratio of 1.58. Once again, a comparison etween washed and unwashed sperm showed that there had been no change

in absorption of the sperm due to the citric acid treatment. Moreover, their absorption was of the same order as that of the Tyrode-washed sperm. The lower ratio obtained in the citric acid experiment was therefore due to an increase in the absorption of the thymus nuclei. Since, however, our ratios

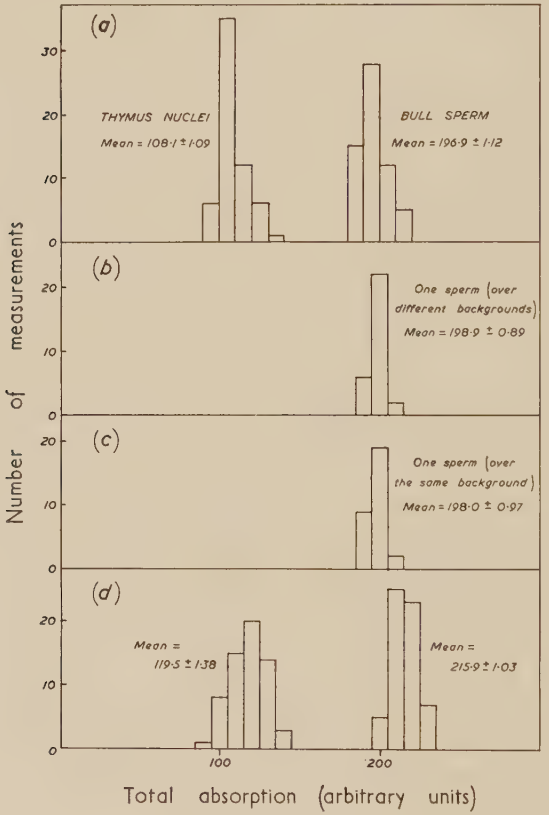


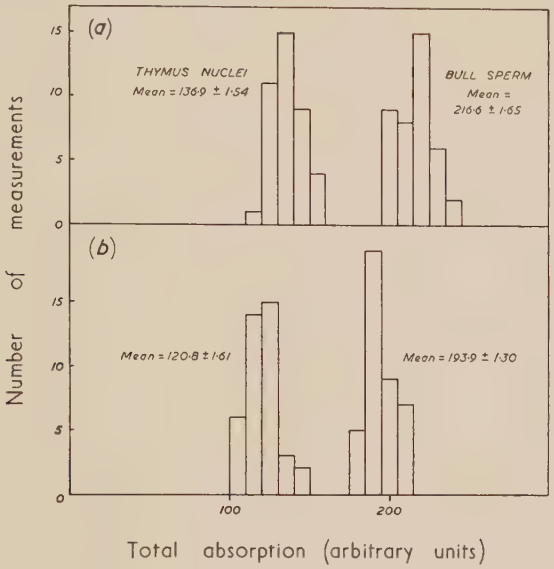
Fig. 1. (*a*) Results of experiment in which Tyrode solution was used for isolating calf thymus nuclei and for washing sperm. (*d*) Repeat experiment using different samples of thymus and sperm. (*b*) Result of 30 successive measurements on the same single sperm with respect to 30 different positions in the clear field. (*c*) Result of 30 successive measurements on the same single sperm selected in (*b*) with respect to one position in the clear field.

were still higher than those obtained by chemical estimation, a further explanation had to be sought.

Vendrely *et al.* state that there is some cytoplasmic contamination in their samples of isolated nuclei and that this may be a source of error. It would in fact, give an overestimate of the amount of arginine per nucleus and in consequence a lower ratio. With the present photometric method it is possible to minimise this error by selecting clean nuclei for measurement and ignoring those which have cytoplasm attached to, or near them. The few large nuclei encountered in our preparations, which had approximately twice the normal

:hount of absorption, were assumed to be polyploid, but these were consid-
[ed infrequent enough to be safely ignored.

An assessment of the true variation of our readings was obtained by a ntinuous series of measurements on one sperm (Fig. 1 *b* and *c*). The main



g. 2. (*a*) Experiment in which *M*/18 ric acid was used for isolating the ymus nuclei and for washing sperm.) Repeat experiment using different mples of thymus and sperm.

rror seemed to be due to minor fluctuations of the recording instrument nd not to differences between various clear field backgrounds. Measurements n single, and on groups of two or more calf thymus nuclei showed that ie readings were proportional to the amount of colour present (Fig. 3). The esult indicated that this instrument is as reliable for this staining technique s it had previously been shown to be for the Feulgen method [3].

In order to determine the amount of fading which occurs with this method, reparations made in the first two experiments (Fig. 1 *a* and *d*) were retained so iat samples of nuclei and sperm could be measured on three consecutive ays. In both experiments almost identical fading curves were obtained and iowed that in both the thymus nuclei and sperm there was approximately per cent loss of colour during the first eight hours. Since the results shown i Figs. 1 and 2 were obtained from measurements made within six hours f making the preparations, fading was not considered to be a serious source f error. Over the following two days however the sperm lost an additional 5 per cent of colour and the thymus nuclei an additional 9 per cent. This ould not have been so serious if the ratio of means had remained the same,

but since the sperm faded more rapidly than the thymus nuclei the ratio had dropped to 1.59 after 48 hours.

Further experiments have been done to determine the effects of the fixation in Lewitsky fluid. Fixation times were extended from 15 minutes to 1 hour,

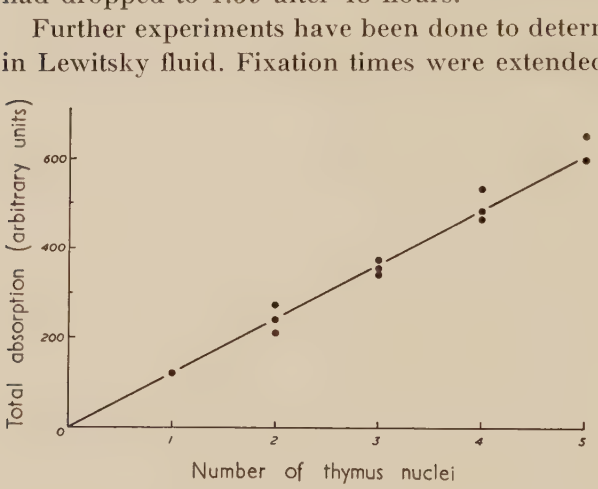


Fig. 3. Relationship between number of calf thymus nuclei (isolated in citric acid) and total absorption.

$2\frac{1}{2}$ hours and 30 hours, Tyrode again being used for isolating the thymus nuclei and for washing the sperm. The mean absorption values from samples of 30 sperm and nuclei are shown in Table I. Although a loss of total absorption was recorded at 1 hour and at $2\frac{1}{2}$ hours, the loss in the sperm was proportional to that in the nuclei so that the ratio of means was not significantly altered. But prolonged fixation led to considerable and unequal loss and thus a drop in the ratio. This may have been due to the action of the formalin.

The method has also been applied with positive results to sections of mouse liver, roots of *Allium cepa* and *Scilla sibirica* and anthers of *Allium schoenoprasum*.

The following factors are being investigated further with a view to making the measurements absolutely quantitative instead of only comparatively

Table I

	Time of fixation			
	15 min.	1 hour	$2\frac{1}{2}$ hours	30 hours
Bull sperm	199.8 ± 1.84	173.8 ± 1.95	144.5 ± 1.63	107.0 ± 1.98
Calf thymus nuclei	106.7 ± 1.88	91.7 ± 1.94	77.4 ± 1.54	75.0 ± 2.48
Ratio	*1.87*	*1.89*	*1.87*	*1.43*

uantitative: (1) The effect of various methods of fixation; (2) the availability l the arginine for the Sakaguchi reaction; and (3) improvements in the kaguchi reaction.

SUMMARY

A modification of the Sakaguchi reaction for arginine, suitable for micro-notometry, is described. Comparisons are made between the results derived y this method and those by standard biochemical procedures.

We should like to thank Professor J. F. Danielli, Dr. Honor B. Fell F.R.S. and r. K. S. Dodds for their helpful criticism. We are also grateful to Professor J. T. andall F.R.S. for his interest and for the provision of working facilities in the heatstone Physics Laboratory, King's College. Thanks are also due to Imperial emical Industries Limited for supplying a sample of 2,4-dichloro-α-naphthol and the Agricultural Research Council's Unit of Animal Reproduction, Cambridge, r samples of living bull sperm.

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CHANGE IN THE PHYSIOLOGICAL STATE OF A CELL POPULATION AS A FUNCTION OF CULTURE GROWTH AND AGE (*TETRAHYMENA GELEII*)[1]

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As discussed briefly in a recent publication [6], the study of growth can be related to the cell in culture at two distinct levels: (*a*) to the isolated cell over the single division cycle; (*b*) to a mass population of cells. In the first instance, interest centers around intrinsic cellular factors which govern growth from one division to another over a period during which the cell is presented with an essentially unaltering environment. In the case of a cell population, the basic concern is with change in cell number over a period equalling the length of many individual division cycles. For the most part, the particulars of just how each individual cell progresses from mitosis to mitosis are only secondarily involved in the measurements.

The study of mass population growth, however, is additionally complicated by the *steady* shift in the environment mediated by the activities of the cell mass itself. As the cell density rises, concentrations of nutrients, end products, etc., change at accelerating rates. The changing relationships between the cell mass and its shifting environment is, to some extent, grossly reflected in the growth pattern of the population as it passes successively through lag phase, logarithmic phase, stationary phase, and finally a phase of decline. Thus the course of culture growth is covered by four main periods defined by the changes in the cell number within the population. Presumably, this indicates the presence of four general states in the physiological interrelationships between cell mass and environment. The acceptance of the foregoing must not obscure the fact that the environment, through activities of the cells, is changed continuously, and undoubtedly the cell population is itself adjusting continuously throughout each phase.

Attention is frequently directed toward the logarithmic phase of growth during which the average generation time remains constant. This apparent

[1] Supported by the American Cancer Society through an Institutional Research Grant to the University of California Medical Center, Los Angeles, California.

teady state" has sometimes been accepted too readily as conclusive evi- nce that the logarithmic phase is physiologically homogeneous, i.e. that a clone the cells from one time point during logarithmic growth are physio- gically identical with all cells from all other time points of this phase.

The experiments to be described here were originally designed to test is assumption using the length of lag phase as a criterion of physiological ate of clonal cell groups with known culture histories. The data, in addition, lp to clarify some of the factors contributing to the existence of the initial g period in mass culture.

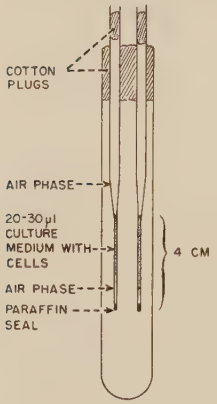
MATERIALS AND METHODS

Cell cultures.—A clone of *Tetrahymena geleii* (HS) [1] was maintained at 25°C in ne per cent proteose peptone buffered at pH 7.4 with 0.009 *M* Na_2HPO_4 and auto- aved ten minutes at sixteen pounds pressure. To provide a large surface to volume tio for rapid gas exchange the cultures were kept in 250 ml Erlenmeyer flasks ntaining 25 ml of nutrient medium. The clone stock culture was renewed every 4 hours by transfer of 0.1 ml inoculum to fresh medium.

Growth measurements.—The objective of the experiments demanded that the rowth curves be precisely defined in order to permit sufficiently accurate dermina- ons of lag phase times under varying conditions. The ideal system would, of course, ermit the counting of every individual cell in a population during the whole course f culture growth. Instead of this, satisfactory estimates of cell number can often e made by such techniques as determination of optical density of the growing culture, etermining the cell count in diluted aliquots of the culture, etc. The method em- loyed here involves working with populations sufficiently small to allow the counting f each living cell but still large enough to give very precisely defined, repeatable rowth curves. The cell counts were made frequently during the first 16 to 24 hours f culture, by which time the curve slope during the logarithmic phase was well stablished. Beyond 24 hours the cell number usually rose too high for accurate ounting.

The details of the method are as follows: *Tetrahymena* with a known culture history vere inoculated into 25 ml of fresh proteose peptone medium (buffered at 7.4) to ive a concentration of approximately 150 cells per ml. From this main flask, aliquots f medium were drawn up aseptically into 12 capillary culture pipettes (1 to 2 mm ore) (Fig. 1). Each pipette contained 20 to 30 μl of medium and 3 to 6 cells. The ips of the pipettes were sealed with paraffin and held in cotton-stoppered, dry test ubes in a constant temperature bath at 25°C ± 0.02°C. During the setting-up of he experiment, which takes about 10 minutes, the room temperature was kept t 25°C and extreme care was taken never to expose the cells to any change in tem- erature. With the above arrangement the total cell number (initially 50 to 60) in bout 350 μl of medium could be easily counted as they increased in number by xamining each pipette under a dissecting microscope (3 × obj., 9 × ocular). The ounting was facilitated by employment of a blue filter and oblique lighting such

that each cell stood out as a bright white spot (usually moving) against a dark background. As the population density approached 100 cells per pipette, the counting became difficult. The experiments were, therefore, discontinued at the end of about 15 hours of logarithmic growth. During this period, the total cell number increased from 50 to 60 up to 900 to 1100. The curves in Fig. 3 attest to the exactness with which generation time during logarithmic growth can be repeatedly determined.



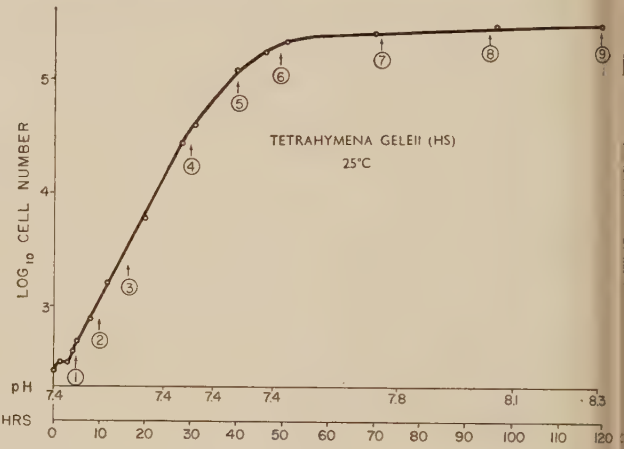


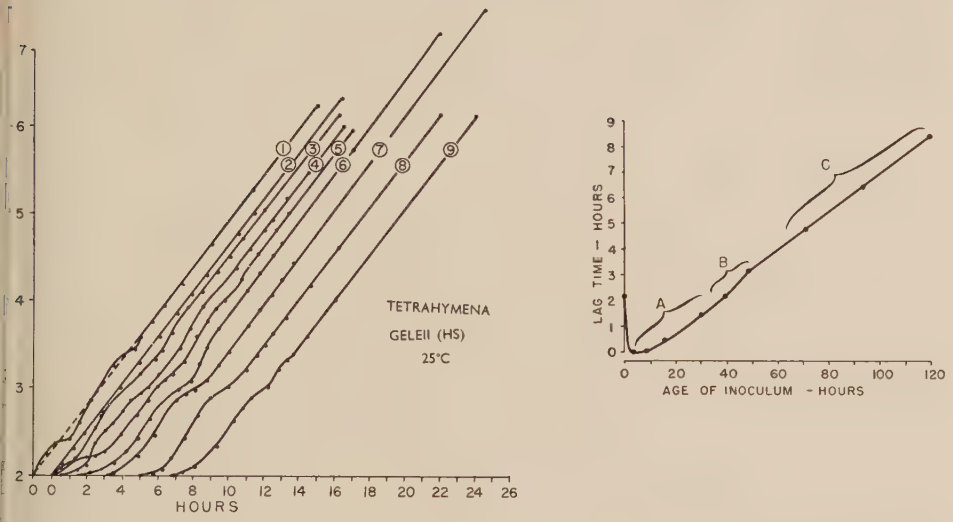
Fig. 1 (*left*). The two culture pipettes are held in a cotton-stoppered, dry test tube immersed in a constant temperature bath at 25°C. For cell counting, each pipette is removed from the tube and examined with a dissecting microscope.
Fig. 2 (*right*). A 120-hour growth curve for a mass culture of *Tetrahymena geleii* (HS) at 25°C. Inocula were withdrawn at time points indicated by the numbers one through nine and lag times determined for cultures initiated with these inocula (see Fig. 3). The pH remains constant until the end of logarithmic growth.

The main theme of the experiments involved the determination of lag times for cultures inoculated with cells taken from various points of culture growth, i.e. inocula of various ages. To relate the inocula ages to phases of culture growth required the determination of a complete growth curve at 25°C. Twenty-five ml of medium in a 250 ml flask were inoculated with 0.1 ml of a 24-hour stock culture grown at 25°C. Absolute accuracy was not so important in this case, and a more usual method of measuring growth could be employed. Ten-μl aliquots were periodically withdrawn from the culture and cell counts carried out on these samples. The 120-hour growth curve in Fig. 2 was obtained in this manner.

EXPERIMENTAL RESULTS

The inocula for the growth curves in Fig. 3 were prepared as follows: One-tenth ml of medium from a 24-hour culture was transferred to 25 ml of fresh medium in a 250 ml flask. The latter was incubated 40 hours at 25°C and a small sample inoculated into a flask of fresh medium to give a final

nsity of approximately 150 cells per ml. This last culture served as the urce of inocula of various ages. The lengths of lag times in Fig. 3 (see also ble I) were obtained by extrapolation of the exponential slope back to e time axis; the difference between zero time and the intercept is considered the lag time. This convenient method of lag-time calculation was suggested



g. 3 (*left*). Nine growth curves for small populations of *Tetrahymena geleii* (HS) at 25°C. For the rious curves, growth was initiated with inocula of different ages (see Fig. 2). Only curves 1 and 2 ve-and ten-hour inocula) evidence no lag phase. To avoid confusion, curves one and two are parated by including two zero-time points on the ordinate. See Table I for further information. g. 4 (*right*). Relationship between ages of inocula and the lengths of lag times. Inocula were taken om cultures in (*A*) logarithmic phase, (*B*) phase of growth deceleration, and (*C*) stationary hase.

y Lodge and Hinshelwood [4] and its advantages are discussed by Monod 5]. The important information derived from the growth curves in Fig. 3 is ummarized in Table I.

Fig. 2 is a 120-hour growth curve at 24°C. Marked along its course are ne points (numbered 1–9) at which the various inocula were withdrawn. hus, curves 1–4 of Fig. 3 were initiated with inocula taken from a logarith- nic phase culture; curves 5 and 6 were initiated with inocula in a state of ecelerating growth; the inocula for curves 7–9 were taken from a stationary hase culture. For convenience of comparison, the growth curves (Fig. 3) are lotted on a percentage basis with the original number considered as 100 per ent. The $\log_2$ of the percentage increase over the original cell number is

TABLE I

The lengths of lag times are listed for cultures initiated with progressively older inocula. The age of the inoculum does not influence the average generation time. The data are derived from Fig. 3.

Culture number	Age of inoculum (hours)	State of inoculum	Average generation time (hours)	Lag time (hours)
1	5	log. phase	3.8	0
2	10	log. phase	3.8	0
3	16	log. phase	3.8	0.5
4	30	log. phase	3.7	1.4
5	40	Growth deceleration	3.7	2.2
6	49	Growth deceleration	3.7	3.2
7	71	Stationary phase	3.7	4.8
8	94	Stationary phase	3.7	6.5
9	120	Stationary phase	3.8	8.5

marked on the abscissa. This simply means that each unit increase along the abscissa represents a doubling in the population, and the time for each doubling corresponds, of course, to the average generation time.

The 0.009 *M* phosphate was sufficient to maintain the pH at 7.4 in the culture used as inoculum source during the first 48 hours of incubation. During the stationary phase, the pH shifted to 8.3 by 120 hours. Times of pH measurements are marked in Fig. 2. Only with inocula from a five-and ten-hour culture is there no lag period before logarithmic growth (see curves 1 and 2, Fig. 3). The relationship between lag time and inoculum age is presented graphically in Fig. 4. The lag times for inocula older than ten hours but still in logarithmic stage increase with inoculum age at a slightly higher than linear rate, while the lag time for an inoculum in stationary phase is directly proportional to the age of the inoculum.

The results of the above experiments suggested that lag time is principally a period of readjustment of the cells to a new environment, and readjustment (conditioning) of the medium during this period is, at most, only slightly involved. The following experiment supports this hypothesis. Twenty-five ml of fresh proteose-peptone was inoculated with 1 ml of a 120-hour culture and incubated at 25°C for twelve hours. The *Tetrahymena* were then concentrated into 1 ml of medium by centrifugation and reinoculated into a second flask of fresh medium. From this last culture aliquots were withdrawn into culture pipettes to permit growth to be followed on an initial count of

) cells. Under these conditions there was no lag period. Apparently, during initial twelve hours the 120-hour cells completely readjusted to the new vironment. The activities of the cells did not significantly change the me- um during this time, for, upon reinoculation into a new supply of fresh dium, no detectable period of readjustment (lag phase) ensued.

t has sometimes been suggested that the lag period may result, in part, m death of some of the newly inoculated cells. No cell deaths were observed any of the above hundreds of lag phase cells; with the optical system em- yed, dead cells would be easily detected. However, in some current experi- nts in which *Tetrahymena* are grown on complete synthetic medium [2] h transfer of cells from stationary phase conditions, pH 8.2, to new syn- tic medium at pH 6.2, approximately twenty per cent of the population olyzes during the initial forty minutes.

As seen from Table I, the average generation time can be determined with nsiderable accuracy; at 25°C the generation time is 3.75 ± 0.05 hours.

During the first doubling of the population on the growth curves in Fig. 3, increase in cell number follows a distinct pattern. In some cases the ttern is repeated in dampened form during the succeeding division cycle. ie events underlying this pattern and its variations are as follows: When ls older than ten hours but younger than seventy-one hours are inoculated o new medium, the ensuing process of adjustment to the new conditions accompanied by the imposition of a small degree of division synchrony. ter all cells in the population have divided once, there follows a period low division index which is a reflection of the initial lag period. A second flection of the lag period is sometimes still perceptible following the second pulation doubling. Because of individual variations in division cycle time, e small degree of synchrony is soon lost and complete asynchrony appears. lls seventy-one hours and older show a slightly different pattern. A distinct flection of the lag period appears after the first cell doubling only, and the pe describing the first population doubling is displaced 0.5 to 1 hour to e left of the straight line phase of logarithmic growth. Apparently, these cells gin division before readjustment to the new medium is completed; as a sult, the division cycle period for the first doubling is 0.5 to 1 hour longer an that known to prevail at this temperature during logarithmic growth.

Occasionally, a few divisions may occur immediately after inoculation into w medium and preceding the lag phase (curve 5, Fig. 3). Probably those lls which have reached a certain proximity to division under the previous lture conditions are capable of completing the preparations for division in ite of the necessity for readjustments when transferred to fresh medium.

Curve 1 in Fig. 3 (five-hour inoculum) evidences a weak rhythmicity but no lag phase. This is to be expected since, in this case, a 40-hour culture served as the inoculum for the culture which in turn furnished the five-hour inoculum. We should, therefore, expect a five-hour inoculum curve to resemble exactly a 40-hour inoculum curve (curve 5, Fig. 3) from which the initial five-hour period has been omitted.

In the determination of lag-phase times, the number of cells in the inoculum is not a critical factor. The lag times in Table I were obtained with initial cell densities of approximately 150 per ml. Increasing this to 400 cells per ml had no detectable effect on the length of lag phase; 1200 cells per ml resulted in a shortening of about ten per cent. This information further supports the hypothesis that the lag phase constitutes a period of readjustment of the cells to the medium without any essential alteration in the medium.

DISCUSSION AND CONCLUSIONS

The experiments demonstrate a quantitative relationship between the culture history of a cell population and the length of the readjustment period (lag phase) imposed upon these cells by transfer to fresh, standard medium. This in itself is not an entirely new observation. The existence of such a relationship has been mentioned previously for *Tetrahymena* [3] and other microorganisms in culture [7]. The very accurate determinations of lag times here, however, permit a much more precise definition of the general events involved and reveal certain specific facts concerning the properties of the cell mass itself.

The operation of two general phenomena underlie the results of the experiments; (1) through the interrelationships between the cell mass and the environment, metabolic activities of the cells continuously affect the constitution of the medium, and (2) reciprocally, changes in the medium influence conditions and events existing within the individual cells, gradually and continuously altering their physiological state. The curve in Fig. 4 describing the relationship between inoculum age and lag time is considered to represent the course and rate of shift in the physiological state of the cell population during culture growth. The criterion of physiological state in this case is the length of time required for the cells to readjust to fresh, standard medium. This same criterion, however, can also be employed as a measure of changes which may have occurred in the culture medium. No readjustment phase could appear unless there were a change in the environment (medium) to force the cells into readjustments. Thus, with the exception of the first few

urs, it seems legitimate to consider the curve in Fig. 4 as also describing the urse and rate of change in the medium as culture growth progresses.

It appears that the lag period is almost entirely one of cell readjustment, th very little alteration in the medium. *Tetrahymena* transferred to fresh edium at the end of lag phase evidence no additional lag phase in the new edium. This indicates that the medium undergoes essentially no change ring the period of cell readjustment. There is, furthermore, an indication at the cells are capable of readjustment at a rate far exceeding the rate of edium shift occurring over the logarithmic and stationary phases of culture. an approximation of this, 120 hours (see Fig. 4) is required for a particular gree of alteration in the medium. *Tetrahymena* are capable of adjusting this same degree of medium alteration in slightly more than eight hours ag time for a 120-hour inoculum). The cells readjust at a rate approximately urteen times the rate of medium shift. In view of this information, cell adjustments must be considered as the dominant factor in governing lag ne length. Finally, in view of the rapidity with which these cells are capable adjusting to changing conditions in the medium, very likely they easily ep pace with those medium changes mediated by the metabolic activities the population itself during culture growth.

When the adjustment between cells and medium is complete, logarithmic owth begins. With the rise in cell density, the state of the medium and cell opulation changes at an accelerating rate. The falling-off of cell multiplication around forty hours marks a decrease in the rate of cell-medium alteration; finally, during the stationary phase, this rate remains constant. The ourse of the curve in Fig. 4 is the result of a composite of such factors as ell density, cell size, the level and character of cellular metabolic activities, ngth of time the cells act on the medium, temperature, etc.

That the logarithmic phase is not a period of physiological homogeneity is learly demonstrated by the increased lag time evidenced by cells from rogressively older logarithmic phase cultures. Cells at the beginning of garithmic growth continue to multiply exponentially when transferred to resh medium. During the late logarithmic stages such a transfer is followed y a readjustment period lasting 1.4 hours or more. Even though the average eneration time remains constant, the constitution of both cells and medium s steadily changing. Obviously, the cells are capable of maintaining a minimum, constant generation time within a certain range of variation in the nvironment. The rate-limiting processes which govern the length of generation time are to some degree, at least, independent of the cell-medium interelationships.

Concerning the basis for the lag period, it is impossible to be more specific until more details of metabolic activities of the cells during this period are known and can be compared to activities during other culture phases. Perhaps, in part, the lag phase represents a period of readjustment in enzyme levels and synthetic pathways.

SUMMARY

By growing small numbers of *Tetrahymena* in culture pipettes, very accurate growth curves are obtainable. This method permits precise determinations of the length of lag phase, which, in turn, can be used as a measure of the physiological state of inocula withdrawn from standard cultures of various ages. The experiments demonstrate a steady shift in the physiological state of the cells and constitution of the medium during the logarithmic and stationary phases of culture growth. The total logarithmic period cannot be considered as physiologically homogeneous even though the average cell generation time remains constant. The lag phase is a period of readjustment to the medium by the newly inoculated cell population. No significant alteration of the medium is detectable during the lag period.

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LA RÉORGANISATION MACRONUCLÉAIRE CHEZ LES *EUPLOTES*

ÉTUDE AU MICROSCOPE ÉLECTRONIQUE

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La structure du macronucleus

Le macronucleus des Ciliés exerce toutes les formes d'activité physiologiques et génétiques d'un noyau normal, mais il se comporte comme un noyau géant, hyperpolyploïde ([14, 20, 24]; voir aussi [10, 11]). L'expérience montre qu'une fraction de ce noyau géant suffit à régénérer un macronucleus génétiquement normal; cette fraction contenait donc au moins un génome complet et l'on doit conclure que de nombreux génomes sont présents dans le macronucleus entier, leur mode de distribution restant indéterminé dans l'état actuel de notre connaissance.

Le macronucleus contient ordinairement deux sortes d'éléments figurés; les uns sont de petits granules riches en acide désoxyribonucléique, fortement colorables par la réaction de Feulgen et par le vert de méthyle; les autres sont les nucléoles contenant des protéines et de l'acide ribonucléique.

Les granules Feulgen-positifs, que l'on peut désigner sous le nom de ADN-microsomes, sont en général de dimension inférieure à 1 μ. Ils sont bien visibles sur les sections ultrafines examinées au microscope électronique [4, 13, etc.]; nous les avons observés dans ces conditions sous des aspects et des dimensions assez variables chez de nombreuses espèces de Ciliés; ils peuvent s'agréger, dans certains cas [18], en globules plus volumineux.

Examinant l'évolution de l'ébauche macronucléaire chez des Ciliés exconjugants, Grell [15] montre que les ADN-microsomes dérivent des chromosomes du syncaryon après que ceux-ci se sont multipliés par endomitose; cependant on ne saurait conclure de ce fait que chaque granule désoxyribonucléique représente un chromosome, une telle correspondance numérique étant probablement faussée par des effets d'agrégation. Les structures filamenteuses observées au cours de la bipartition amitotique du macronucleus ne sont certainement pas chromosomiques; il apparaît d'ailleurs que la méiose

du micronucleus et les divisions post-gamiques du syncaryon seules, laissent voir de véritables chromosomes; et que ceux-ci sont de très petite dimension [6, 7, 21].

La masse du macronucleus s'accroît pendant l'interscission; le nombre des génomes présents doit doubler pendant cette période, sous la réserve hypothétique que de lentes modifications de la balance génique pourraient être responsables du vieillissement de ce noyau somatique [11, 25]. On peut donc supposer que la multiplication par endomitose des structures chromosomiques, observée par Grell au cours du développement de l'ébauche macronucléaire, se continue au cours de l'accroissement et de la bipartition amitotique de ce noyau.

D'une manière générale les aspects présentés par les ADN-microsomes, soit pendant l'interscission, soit pendant la bipartition, n'évoquent pas cette multiplication chromosomique probable; dans certains cas cependant, un aspect structural temporaire, connu sous le nom de « bande de réorganisation », pourrait être considéré, ainsi que le suggère Sonneborn [22], comme l'image d'une « onde d'endomitose ».

Le problème des « bandes de réorganisation »

Le macronucleus allongé en forme de boudin de divers Ciliés Hypotriches et Oligotriches montre, pendant l'interscission, deux bandes claires transversales qui, tels deux septa transparents, le cloisonnent et le partagent en trois compartiments distincts, l'un central, les deux autres terminaux. Chez les *Euplotes*, p. ex. [16, 27, 29], ces bandes claires apparaissent simultanément à chaque extrémité du macronucleus et se déplacent lentement en direction centripète jusqu'au moment où elles se rencontrent pour s'évanouir aussitôt. Chez les *Aspidisca* par contre [26] ces bandes apparaissent dans la région centrale du macronucleus et se déplacent en direction centrifuge vers les deux extrémités où elles disparaissent. Dans l'un et l'autre cas chaque bande parcourt, avant de disparaître, la moitié de la longueur du macronucleus dont elles occupent, l'une et l'autre, toute la largeur; il semble donc que la masse des éléments nucléaires : granules désoxyribonucléiques et nucléoles, soit traversée, dans sa totalité, par les deux septa apparemment vides.

Les « fentes nucléaires » décrites dans les macronuclei ovoïdes de quelques Hypotriches et dans l'orthomère du macronucleus complexe des Dysteriens et des Chonotriches [12] correspondent à un processus analogue.

Considérant le sens du déplacement des bandes claires transversales, on peut définir, pour chacune d'entre elles, un front antérieur (celui qui avance) et un front postérieur (celui qui recule). Le front antérieur est une surface

ane nettement marquée; chez *Euplotes*, Griffin [16] montre que celle-ci nite un plan colorable, basophile, d'aspect homogène épais, de 1 à 2 μ; urner [27] précise que, observé *in vivo*, ce plan est réfringent et que, après ation, il se colore fortement par la réaction de Feulgen en gardant son aspect omogène. A ce plan basophile fait suite un plan de faible indice de réfrac-on, non ou très faiblement colorable, limité par la surface postérieure; lle-ci est moins nettement marquée que la première.

Griffin propose une ingénieuse hypothèse pour interpréter ces aspects; au veau du front antérieur les granules chromatiques — c'est-à-dire les ADN-icrosomes — se dissoudraient dans la caryolymphe en formant le plan olorable, ou « solution plane »; peu après la substance chromatinique se récipiterait en nouveaux granules au delà du front postérieur, ou « reconsti-ution plane ». La bande claire, considérée dans son ensemble apparaît nsi comme une zone de réorganisation du matériel nucléaire. Turner pense ue de tels changements d'état des constituants nucléiniens du macronucleus upposent leur nature colloïdale et la possibilité de transformations réversibles ar dispersion et agrégation.

Cette interprétation physico-chimique des « bandes de réorganisation » et e leur déplacement énigmatique à travers toute la masse du macronucleus, e tient pas compte de l'existence possible et probable de structures chromo-omiques douées d'une certaine permanence et de quelque stabilité. A cet gard on doit retenir la suggestion formulée en ces termes par Sonneborn 23] : « Without wishing to make a dark problem darker, one may at least aise the question as to whether the 'progression' of the reorganisation band ay not represent a wave of mitoses passing through successive component ubnuclei along the length of the macronucleus. »

Les processus macronucléaires de l'interscission en fonction du temps

La succession chronologique des processus macronucléaires au cours de interscission a été examinée chez *Euplotes eurystomus*. Différents individus nt été isolés d'une même culture entretenue avec des *Chilomonas*, et placés hacun dans une lame creuse avec un excès de nourriture. Nous avons d'abord déterminé la durée de l'interscission en plaçant l'ensemble de ces lames dans ne étuve à microscope réglée à 28°C, et en notant pour chaque individu, u cours de fréquents examens, le moment de la première bipartition et elui de la seconde.

A la température de 28°C, l'intervalle entre deux divisions varie de 22 à 24 eures environ, des écarts atteignant ainsi jusqu'à 8% pouvant être constatés ntre deux individus frères.

En dépit de ce degré d'incertitude, nous avons fixé différents individus à des temps qui, comptés à partir de la première division, s'échelonnerait entre une heure et 23 heures environ; ces individus ont été colorés soit par la réaction de Feulgen, soit par le vert de méthyle-pyronine. Pour comparer les résultats nous avons pris comme unité la durée moyenne aproximative de l'interscission, soit 23 heures, et exprimé les temps de 0 à 1, en pour cent de cette durée.

Entre les temps 0 et 0.2, le macronucleus est rempli de granules Feulgen positifs, colorés en bleu vert par le mélange vert de méthyle-pyronine; peu après le temps 0.2, des nucléoles pyroninophiles apparaissent entre les ADN-microsomes; ceux-ci deviennent moins réguliers et quelques-uns semblent s'agglomérer en globules après le temps 0.3. Entre 0.25 et 0.4 le macronucleus d'abord en forme de C, s'allonge de près de 20% et se replie en Σ; il revient ensuite à la longueur initiale.

Les bandes de réorganisation apparaissent à chaque extrémité entre les temps 0.4 et 0.5, soit peu avant la moitié de l'interscission; leur déplacement centripète est d'abord très lent, et les deux parties terminales du macronucleus ne comptent encore ensemble, vers le temps 0.6, que pour 11 à 12% de la longueur nucléaire totale; ces parties s'élargissent légèrement en forme d'olive, et montrent des granules désoxyribonucléiques relativement gros. Le déplacement des bandes s'accentue bientôt et l'on note pour la longueur des deux segments terminaux ensemble 56% de la longueur nucléaire vers le temps 0.74, 86% vers 0.77, 100% vers 0.80. Au cours de cette période, les ADN-microsomes de la partie centrale montrent une disposition réticulée, tandis que dans les segments terminaux ils s'agglomèrent en globules très fortement colorables par le vert de méthyle; les nucléoles pyroninophiles semblent avoir disparu.

Lorsque la « réorganisation » nucléaire est totale, les deux bandes ayant disparu après leur jonction, le macronucleus commence à se raccourcir et à s'épaissir vers le temps 0.83 en prenant un aspect fusiforme; ce stade est de quelque durée et c'est tout près du temps 1.00 que le macronucleus s'allonge en forme de biscuit aux extrémités renflées et se divise rapidement. Pendant ces derniers stades, les granules chromatiniques s'agrègent en travées noduleuses réticulées, étirées par la bipartition.

La structure submicroscopique des « bandes de réorganisation »

Les sections d'*Euplotes eurystomus* fixés au liquide chromo-osmique selon Dalton–Weiss et inclus au *n*-butyl-méthacrylate ont été examinées au micro-

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Les micrographies électroniques du macronucleus montrent des corpus- les de contour plus ou moins régulier, mesurant près de 1 μ ou parfois vantage et correspondant aux granules désoxyribonucléiques colorables r la réaction de Feulgen; les sections perpendiculaires à une « bande de organisation » offrent un aspect général exactement comparable à celui nné par l'examen microscopique à faible grossissement (Fig. 1 et 2), à ci près que le changement d'échelle permet de pousser plus loin l'analyse ucturale.

Le plan colorable de la « zone de réorganisation » apparaît comme un utrage assez lâche et régulier de trabécules ou de filaments plus ou moins ntournés, dont le diamètre est d'environ 35 mμ (Fig. 2, 3, 4, 5, *Ch*). Sur le ont antérieur du plan colorable, ce feutrage est en continuité avec la face stérieure, en voie de désagrégation, des granules désoxyribonucléiques imaires immédiatement adjacents.

Au niveau de son front postérieur, ce même plan colorable se disloque, u fait de l'agrégation de ses constituants filamenteux en petits amas denses, rrespondant à de nouveaux ADN-microsomes; ce processus de condensation isse un espace apparemment vide (Fig. 1 et 2, *c*) qui représente le plan on colorable de la « bande de réorganisation ».

Il apparaît ainsi qu'il n'y a pas, à proprement parler, *dissolution* des élé- ents figurés du macronucleus au niveau de la bande transversale; et que soi-disant « solution plane» résulte de la dispersion des constituants bmicroscopiques des ADN-microsomes. Un complément d'information st ici nécessaire.

A un plus fort grossissement on observe dans les ADN-microsomes en voie e désagrégation, la présence de constituants très petits, dont le diamètre, férieur à 10 mμ paraît être de l'ordre de 80 Å; ces constituants ne sont pas anulaires; ils paraissent allongés, diversement incurvés, peut-être spiralés Fig. 3 et 5, M_1); leur diamètre est nettement inférieur à celui des filaments ont le feutrage constitue le plan colorable. Mais ces derniers apparaissent ux-même hétérogènes (Fig. 5, *Ch*), comme s'ils étaient constitués par des laments ténus, de diamètre légèrement inférieur à 100 Å, contournés ou piralés dans une substance matricielle moins dense.

S'il en est bien ainsi, la dispersion des constituants submicroscopiques des ADN-microsomes s'accompagnerait d'une certaine organisation impliquant n degré de complexité plus élevé que celui d'un changement réversible l'état colloïdal.

DISCUSSION

La microscopie électronique vérifie que les « bandes de réorganisation » macronucléaires des *Euplotes* sont le lieu d'une désagrégation puis d'une reconstitution des granules désoxyribonucléiques; cette transformation qui intéresse progressivement toute la masse des ADN-microsomes, met en évidence des structures submicroscopiques dont il reste à examiner la signification possible. Sachant que cette transformation paraît être liée à l'accroissement de la masse du macronucleus, la question se pose de savoir dans quelle mesure les aspects observés peuvent s'accorder avec l'idée de structures chromosomiques et d'endomitose.

Les plus petits éléments figurés apparaissent dans les granules désoxyribonucléiques lorsque ceux-ci se gonflent avant de se désagréger au niveau du « solution plane ». Il s'agit, comme on l'a vu, de structures apparemment filamenteuses, contournées ou spiralées, dont le diamètre, légèrement inférieur à 10 mμ, paraît être d'environ 80 Å; ces dimensions minimes évoquent l'idée des microfibrilles chromosomiques observées chez des Métazoaires [19, 1, 2, 3, 22], bien que celles-ci soient généralement plus épaisses, leur diamètre étant de l'ordre de 100 à 200 et même 250 Å. Chez d'autres Ciliés, des particules de près de 100 Å ont été déjà observées dans les corps correspondant aux ADN-microsomes (Randall et Filton-Jackson, communication personnelle).

On peut se demander si de telles structures ont une existence propre ou si elles ne sont pas le fait d'une précipitation, par le réactif fixateur, des macromolécules nucléoprotéiques; les dimensions de ces dernières ne leur sont pas très inférieures si l'on considère que, pour l'acide désoxyribonucléique seul, la double hélice polynucléotidique proposée par Watson et Crick [28] accuserait déjà un diamètre de 20 Å (voir [17, 5]). Cependant, la présence

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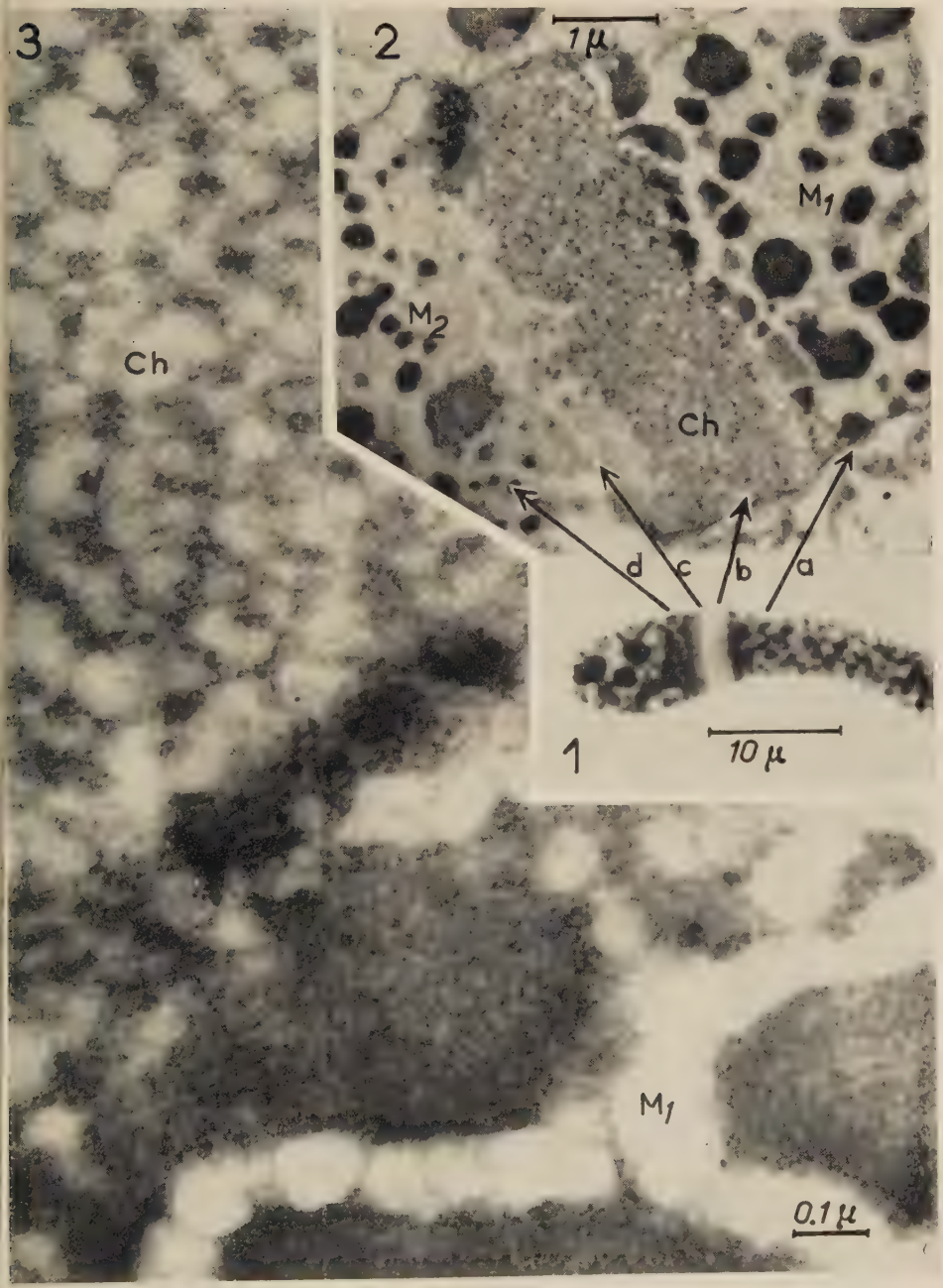
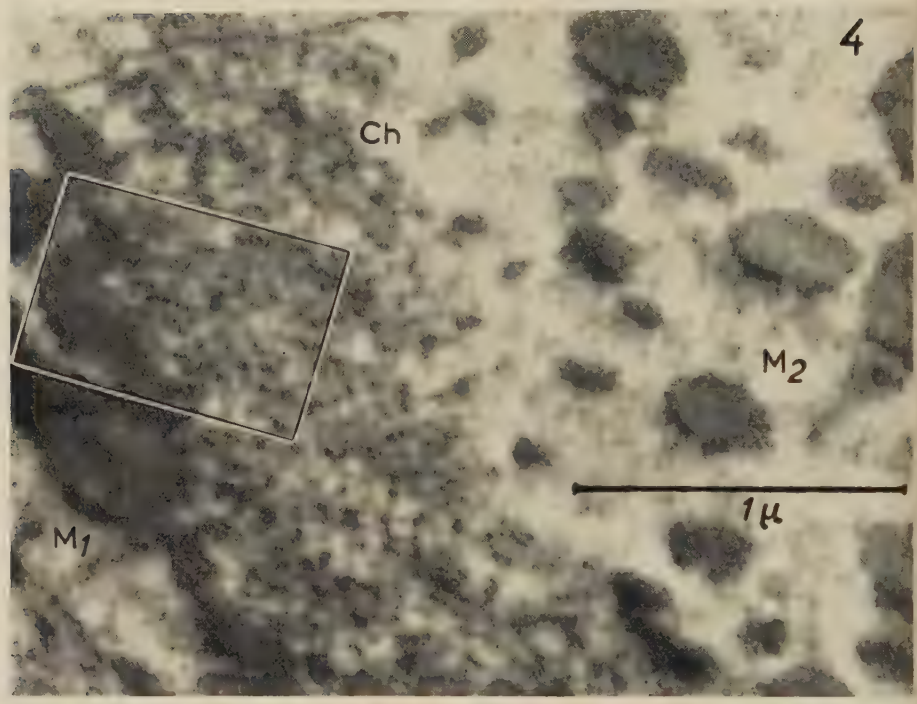
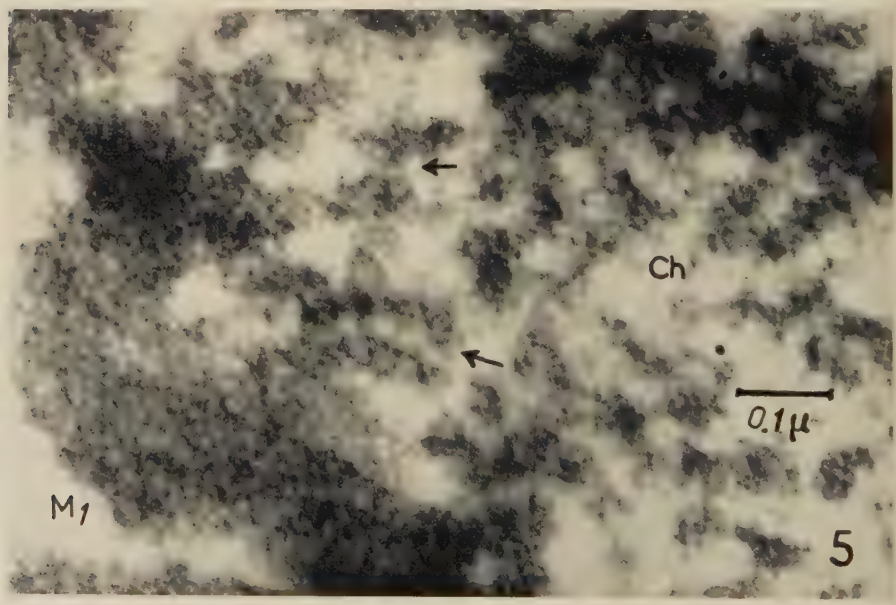
Fig. 1. Microphotographie d'un macronucleus *d'Euplotes eurystomus* en interscission montrant une « bande de réorganisation ». Coloration de Feulgen. Comparer avec la micrographie électronique de la figure 2, les flèches indiquant les zones correspondantes. *a*, Segment central avec les ADN-microsomes primaires; *b*, plan basophile, apparemment homogène; *c*, plan non colorable; *d*, segment terminal avec les ADN-microsomes secondaires (reconstitués).

Fig. 2. Micrographie électronique d'une section passant par une bande de réorganisation. Fixation chromo-osmique. Les flèches marquent les correspondances avec la microphoto Fig. 1. Les ADN-microsomes primaires M_1 se désagrègent sur le front antérieur de la bande en donnant la masse trabéculaire *Ch* correspondant au plan basophile *b*; les trabécules *Ch* s'agrègent sur le front postérieur de la bande en donnant les AFN-microsomes secondaires M_2 dont la condensation provoque la formation de l'espace clair *c*.

Fig. 3. Micrographie électronique d'une section passant par le front antérieur d'une bande de réorganisation. M_1, ADN-microsomes constitués par un feutrage de fins filaments contournés. *Ch*, trabécules chromatiques formés par la dispersion des filaments contournés.


3
2
1 μ
M1
M2
Ch
Ch
d
c
b
a
1
10 μ
M1
0.1 μ


4
Ch
M2
1μ
M1


Ch
0.1μ
M1
5

structures analogues dans les filaments plus épais dont le feutrage constitue lan colorable de la bande de réorganisation laisse supposer que les ADN-rosomes sont constitués par une dense agglomération d'unités submicro-piques filamenteuses possédant déjà une structure complexe multimolé-aire. L'état d'agrégation de ces unités peut varier soit au cours du cycle siologique de croissance nucléaire, soit sous diverses conditions expéri-ntales ou naturelles; on sait en effet que l'on peut modifier réversiblement structure granuleuse d'un macronucleus isolé en fonction du pH du milieu érieur [8] et que, chez les Ciliés vivant ordinairement dans un milieu forte concentration saline, le macronucleus paraît optiquement vide à clairage sur fond noir, mais devient granuleux si l'on dilue le liquide exté-ur [9]; ces faits avaient été interprétés par des changements de l'état loïdal des nucléoprotéines, c'est-à-dire suivant le même schéma que celui posé par Griffin et par Turner pour expliquer les "bandes de réorganisa-n" chez les *Euplotes*.

Les trabécules dont la masse feutrée constitue le « solution plane », appa-ssent, malgré leurs petites dimensions, beaucoup moins comme un préci-é colloïdal que comme des structures organisées. Il est évidemment très ficile de décider si l'on est en droit de les comparer à des éléments chromo-miques; la possibilité d'un processus de duplication est évoquée par cer-nes images (Fig. 5) montrant deux segments fibrillaires parallèles. Aucune nclusion positive concernant le processus d'endomitose ne peut, cependant, e apportée par ces observations.[1]

SOMMAIRE

L'examen au microscope électronique des « bandes de réorganisation » ns le macronucleus d'un *Euplotes* montre la dispersion de la substance des anules désoxyribonucléiques primaires sous formes de très fins filaments,

[1] Un abstract du travail de L. E. Roth, « Further electron microscope studies of *Euplotes tella*, paru dans le supplément du *J. Protozool.* **3**, 5 (1956), indique: "In the macronuclear re-ganization bands, the solution plane has a structural appearance similar to the chromatin dies.»

g. 4. Micrographie électronique d'une section de bande de réorganisation. M_1, ADN-microsomes imaires en désagrégation sur le front antérieur de la bande; *Ch*, trabécules constituant le plan sophile; M_2, ADN-microsomes secondaires reconstitués sur le front postérieur de la bande. e cadre correspond à la figure 5.

g. 5. Partie de la figure 4 (marquée par le cadre) plus fortement grossie. M_1, ADN-microsome imaire dont les constituants filamenteux et contournés sont en voie de dispersion sous forme trabécules *Ch* à structure spiralée. Noter d'après les flèches que les trabécules sont peut-être sposés par paires.

puis l'agrégation de ces filaments et la formation de granules désoxyribonucléiques secondaires.

La nature des structures filamenteuses et trabéculaires observées est discutée en relation avec l'hypothèse d'un processus endomitotique.

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CYTOCHEMICAL STUDIES OF FERTILIZATION AND FIRST MITOSIS OF THE SEA URCHIN EGG

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ıE structural differences of the cytoplasm of mature unfertilized as well fertilized eggs were studied by Runnström [19, 20] both in living and fixed ıterial. The differences described by Runnström are conspicuous also in ɔparations where the yolk has been electively stained. In the unfertilized ɡs the yolk globules are more irregularly distributed than in the fertilized ɡs, cf. Monné and Hårde [17]. The structure of the ground cytoplasm is ɔre homogeneous and the yolk is more intensely stained by means of the riodic acid–Schiff technique (PAS) in fertilized than in unfertilized eggs. ıis statement concerning eggs of *Paracentrotus lividus* was shown by the ıesent writer to hold also for eggs of *Arbacia lixula, Echinus esculentus, ammechinus miliaris* and *Strongylocentrotus droebachiensis*. At the same time the distribution of the yolk granules changes upon fertilization their stain-ɡ with PAS undergoes a conspicuous increase.

The presence and location of polysaccharides in the mitotic figure is the ain subject of the present paper.

McManus [15] stated that human mitotic figures, especially in tumors, give positive reaction with PAS. Monné and co-workers [18] found that dividing a urchin eggs contain polysaccharides within the spindle, particularly at e spindle poles and occasionally they may appear also in the chromatin the interphase nucleus [17, 18]. According to Stich [21, 22] the larger part polysaccharides present in the nuclear sap of oocytes or eggs of *Cyclops renuus* enters as essential components of the fibrils of the division spindle. azia and Dan [12, 13, 14] have isolated the mitotic apparatus of two spe-es of *Strongylocentrotus*. Later Mazia found a positive PAS reaction in this pparatus.

The possible causes of the changes in the PAS reaction upon fertilization ıd during mitosis will be discussed. Beside the main subject of this paper ɔme observations will also be described which refer to the Feulgen staining f the male and female pronucleus.

MATERIAL AND METHODS

Most observations of the present investigations were carried out on eggs of *Paracentrotus lividus*, fixed in Carnoy's fluid as previously described [4]. The fixation was carried out with intervals of five minutes from insemination until the two-cell stage. Thus twenty various development stages were fixed and examined. Microscopical technique and staining methods used was the same as in previous papers [4, 5]. Control sections treated according to McManus' [16] acetylation technique confirm that the aldehyde radicals are obtained from the hydroxyl groups of carbohydrate in α-glycol linkage.

RESULTS

The number of aldehyde groups produced by periodic acid oxidation in the mucopolysaccharides of the yolk granules increases immediately after fertilization (Fig. 1). Thereafter the intensity of the aldehyde reaction seems to be constant at least until the two-cell stage.

The nucleus of spermatozoön always gives a positive Feulgen reaction. Conversely, this reaction proved to be negative in the nucleus of mature eggs of *Echinus esculentus, Paracentrotus lividus, Psammechinus miliaris* and *Strongylocentrotus droebachiensis*. The male nucleus penetrates into the female nucleus 30 minutes after fertilization. In this phase the male nucleus is seen clearly as a Feulgen positive body within the female nucleus in which no desoxysugars are demonstrated (Fig. 3*d*). Forty minutes after fertilization the male nucleus is visible in the form of a group of small granules (Fig. 3*e*). Forty-five minutes after fertilization no positive Feulgen reaction can be demonstrated in the syncarion (Fig. 3*f*), whereas in the meta- and anaphases (70 minutes after fertilization) the positive reaction reappears, now located in the chromosomes (Fig. 3*g*, *h*). In telophase the positive Feulgen reaction dis-

Fig. 3. Scheme of the Feulgen reaction of the nuclear components of the pronuclei, of the syncarion and during the first mitosis. *a*, unfertilized egg; *b*, 15 minutes; *c*, 25 minutes; *d*, 30 minutes; *e*, 40 minutes; *f*, 45 to 70 minutes: *g* and *h*, 70 to 75 minutes; *i*, 85 minutes after fertilization according to observations on *Paracentrotus lividus*.

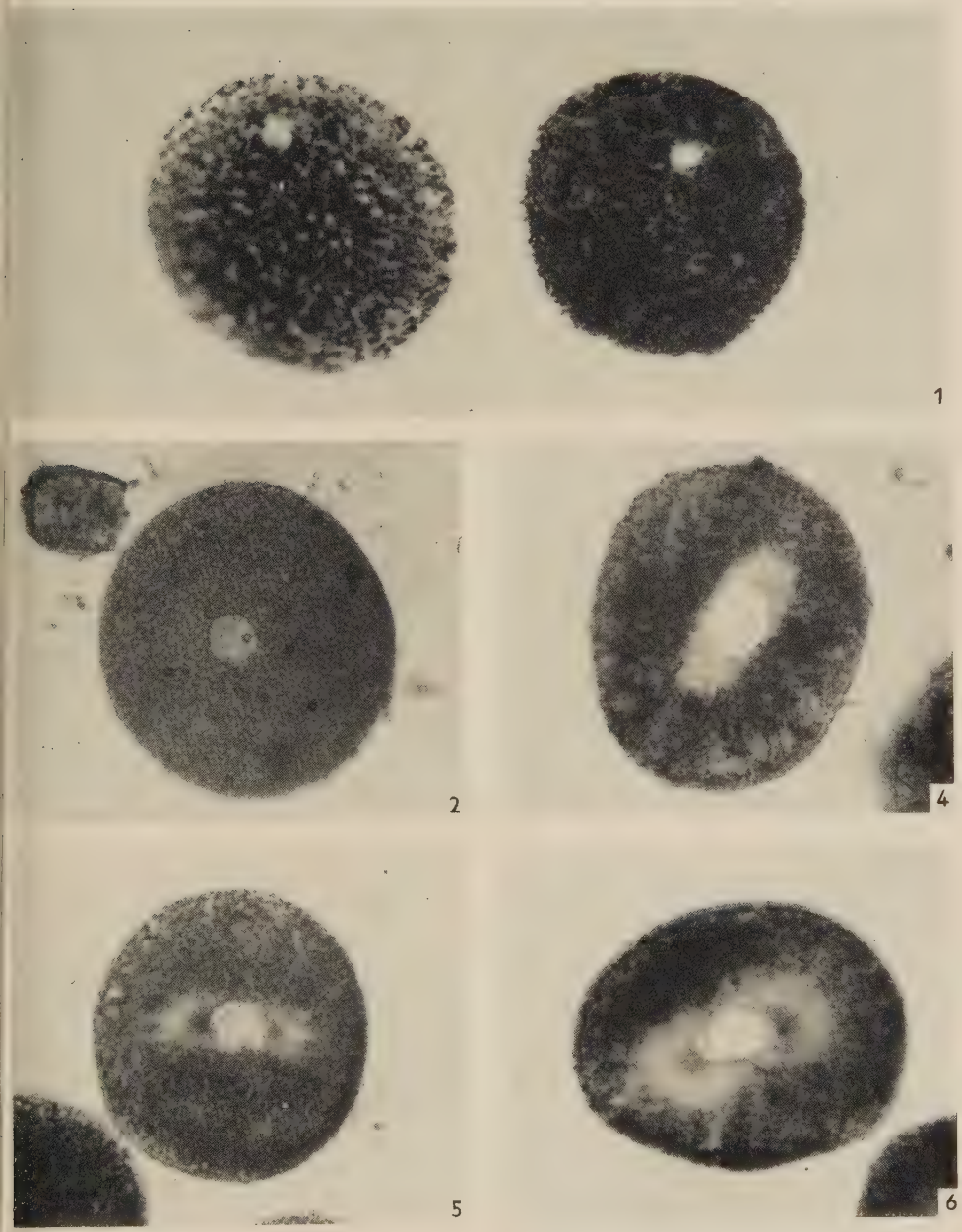

Fig. 1. *Echinus esculentus* mature unfertilized egg (left) and fertilized (right), 10 minutes after fertilization. PAS-staining. Photographed with Ilford light-filter No. 408 (green).
Figs. 2, 4, 5, 6. *Paracentrotus lividus*, fertilized eggs, stained and photographed as Fig. 1. *Fig. 2*, 5 minutes; *Fig. 4*, 30 minutes; *Fig. 5*, 50 minutes; *Fig. 6*, 60 minutes after fertilization.

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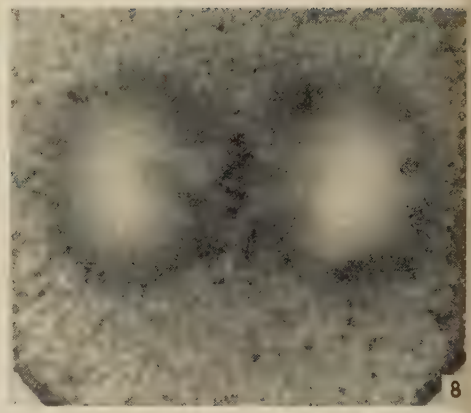
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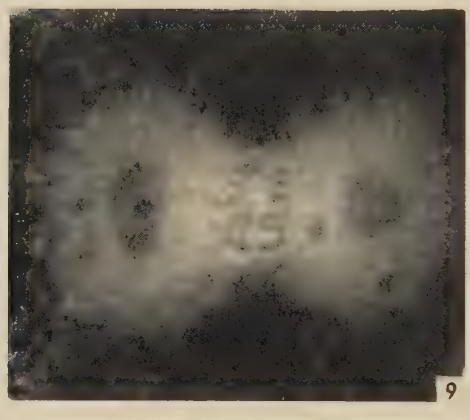
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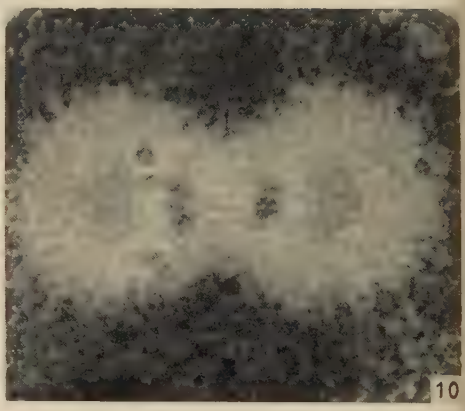
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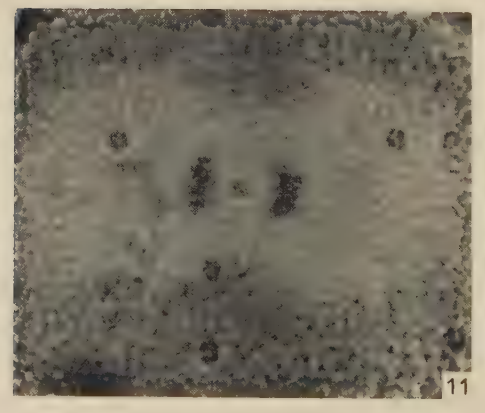
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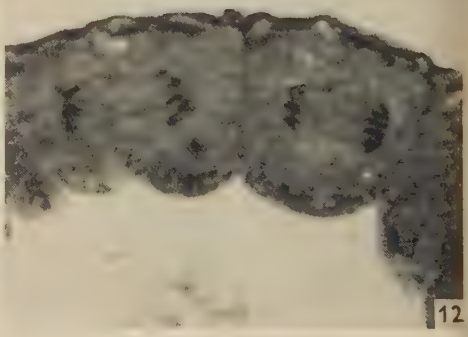
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pears and is also absent in the resting nucleus of the two-cell stage but m the four-cell stage on, the reaction in the resting nuclei is positive.

n the early stages after fertilization the yolk granules are rather uniformly tributed over the cytoplasm (Fig. 2). In the stage 25 minutes after fertiliza- n the yolk granules begin to retreat from the sphere of the cytoplasm sur- nding the interacting male and female nuclei. In the further development cytoplasmic sphere, devoid of yolk granules, increases to an elipse with longer diameter perpendicular to the future first cleavage plane (Fig. 4). e two opposite spindle poles appear near the syncarion 50 minutes after tilization. The cytoplasm of the spindle poles is intensely PAS positive g. 5). The color intensity of the spindle poles increases and obtains its ximum in the prophase (Fig. 7). Then the intensity decreases gradually igs. 7, 9, 10) and the color completely disappears in the telophase. The yolk nules displaced from the cytoplasmic sphere are concentrated and ac- mulated in the equatorial region of the cytoplasm surrounding the division ndle (see Fig. 7). In the prophase (spireme Fig. 6, transition from prophase metaphase Fig. 7) a very weak positive PAS reaction was obtained in the clear substances. In the meta- and early anaphases the nuclear material ring a positive PAS reaction is located in the chromosomes (Fig. 9). In e anaphase as well as in telophase the chromosomes are PAS-negative, hough they are clearly stained with Gentian violet stain (Fig. 12).

The Hale ferric iron reaction on phosphate groups is occasionally faintly sitive in the prophase but always distinctly positive in meta- and anaphase romosomes (Figs. 10, 11). In telophase the reaction is definitely negative.

With methylgreen–pyronin the chromosomes stain bluish-green in meta- d anaphases but pale red in pro- and telophases. Also the hyaline layer ains bluish-green.

In all stages of mitosis the chromosomes stain magenta with azur–eosin- ethylene blue (Giemsa method); the spindle fibers on the other hand, main colorless (Fig. 8). The nuclei of resting cells do not stain with this ethod.

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gs. 7–12. Mitotic figures of *Paracentrotus lividus* eggs. *Fig. 7*, transition stage from prophase to taphase, PAS-staining, photographed with Ilford light-filter No. 408; *Fig. 8*, metaphase, romosomes are stained magenta by Giemsa method, photographed without filter; *Fig. 9*, aphase, stained by PAS and photographed with light-filter No. 408; *Figs. 10* and *11*, anaphase, thout light-filter (Fig. 10) and with light-filter No. 205 (Fig. 11); *Fig. 12*, late anaphase of late astula cells, stained with Gentian violet and photographed without filter.

DISCUSSION

The presence of DNA in the nucleus of the mature sea urchin egg ha recently been subject to discussion. According to Marshak and Marshak [9 the nucleus of mature eggs contains no detectable Feulgen-positive materia Burgos [1] on the other hand has recently published observations accordin to which the nucleus of mature unfertilized eggs of *Arbacia punctulata* give a positive Feulgen reaction and hence contains DNA. The observation Burgos may be correct, Marshak and Marshak [11] have, however, alread raised well founded objections against his method.

In unpublished work the present writer found that the acetone pretreatment use by Burgos is insufficient to remove disturbing lipids quantitatively. The comple extraction with acetone in a Soxhlet apparatus removes for example only 17.5 pe cent of the substances present in dried eggs of *Arbacia lixula*. It may be added tha the methanol extracts contain about twice as much lipid substances as those prepare with hot acetone, but no breakdown products of DNA were found even after hy drolysis of the extracts with weak hydrochloric acid. In chromatograms of the extracts five different reducing components were found, one of which was identifie as glucosamine. The presence of desoxyribose and ω-hydroxylaevulinic aldehyd could be excluded.

As shown above in four different species of sea urchin the female nucleu shows a negative Feulgen reaction during the first thirty minutes after fe tilization, whereas in the penetrating male nucleus this reaction is strongl positive. It is hardly convincing to assume that the failure of the Feulge reaction in the case of the premitotic female nucleus depends on a state dilution or of fine dispersion of DNA. The extraction experiments referred above as well as the observations made by Marshak and Marshak on th nuclei of centrifuged mature *Arbacia* eggs tend to show that DNA is n present in the female nucleus or at least it is not present in its ordinary form The negative results obtained after staining with methylgreen–pyronine with Hale's acetic iron reagent confirm also this conclusion. It is interestin that also the male nucleus loses for some time its Feulgen positive reactio This is after the stage of "granulation" of the male nucleus, cf. Fig. 3*e*, The stage *f* indicated in this figure lasts for about 25 minutes at room tem perature (20°C) in the eggs of *Paracentrotus lividus*, cf. legend of Fig. 3. Th Feulgen reaction (as well as positive methylgreen and Hale staining) reappear when the metaphase chromosomes are formed.

The presence of PAS positive material in the spireme stage and in th

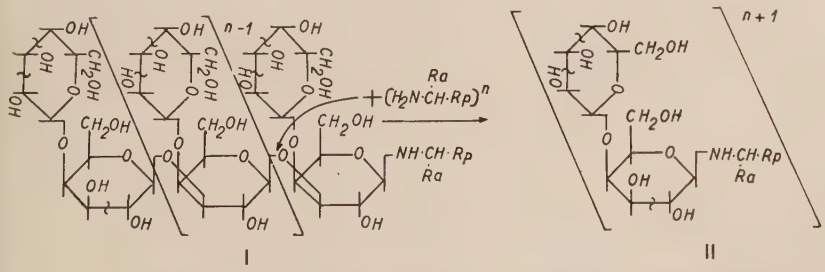
omosomes of the meta- and anaphases as well must be due to the presence unsubstituted α-glycol groups. The reaction is blocked by the McManus tylation technique. Usually the carbohydrate components of RNA and A and also the hydroxyamino acids are chemically substituted, so that no e α-glycol groups are available. The positive reaction in our particular e could possibly be due to hydroxylysine but this amino acid is absent the egg of *Paracentrotus lividus*, cf. Kavanau [6, 7].

A particularly interesting observation was made by McManus [15], viz. t the PAS positive reaction is obtained at the site of alkaline phosphatase. is favors the assumption that under the action of active phosphatase α-glycol ups may be formed in the 2,3 position of the ribose molecule.

The appearance of a positive PAS reaction at the spindle poles (Figs. 5–7) es an indication of structural changes occurring in carbohydrate compo- nts. Presumably these are present in earlier stages in a form similar to t in agar or in the jelly coat of the sea urchin, i.e. the monoses are linked ough 1,3 positions.

In the jelly coat of *Echinocardium cordatum* the monosaccharide units are fuco- anoses, cf. Vasseur [23]. The polysaccharide of the egg cytoplasm, on the other nd, are aldohexoses in the pyranose form [2]. Although the monosaccharide units different, the general outline of the polysaccharide component may be similar, certain polysaccharide components of the egg may thus have a similar comb-like ucture as that ascribed by Vasseur to the main polysaccharides component of the y coat of *Echinocardium*, cf. the adjacent scheme I, Fig. 13. In the main chain linkages are between C_1 and C_3 with branching points at C_4. The point of attack PA are indicated with the wavy line, the protein is supposed to be linked at C_1 an end hexopyranose of the main chain.

The writer would suggest as an hypothesis that the PAS-positive stage develops as onsequence of a rearrangement outlined in scheme II of Fig. 13. The 1,3 linkages broken up and the split products react with proteins. The result is thus a depoly- rization of the polysaccharide and an increased linkage of the split products with oteins. By this change an increased number of α-glycol groups become available the action of PA.



. 13. Hypothetical scheme of mucopolysaccharide; I before and II after fertilization.

It has been shown above that the yolk granules after fertilization become more strongly PAS-positive than before fertilization. This again must mean an increase of available α-glycol groups probably connected with depolymerization and increased linkage with proteins. Fertilized and unfertilized eggs were oxidized by PA for 5, 10 and 15 minutes. There was no progressive increase of the aldehyde groups. The first five minutes period of PA action is sufficient to oxidize all the available α-glycol groups.

Lindberg [8] has provided some evidence that the so-called desmoglycogen fraction—which probably also contains mucopolysaccharides—increases upon fertilization. His data tend to show that upon fertilization the free glycogen is linked to protein and then broken down. The views expressed by Lindberg [8] are in good agreement with the hypothesis suggested above, cf. Fig. 13.

There must occur some rather profound changes in the carbohydrates following fertilization, particularly with respect to the reaction between carbohydrates and proteins. These changed relations may contribute to the important structural and metabolic changes which occur upon fertilization.

SUMMARY

It has been demonstrated that the yolk granules of the sea urchin eggs give a more intense PAS reaction following than prior to fertilization. A hypothetical scheme has been proposed which accounts for the increase of α-glycol groups occurring upon fertilization (cf. scheme I and II, Fig. 13).

The Feulgen staining of the pronuclei and of the syncarion has been studied. The results are given in Fig. 3. They confirm that DNA at least in its ordinary form is absent in the female pronucleus and during a certain period in the syncarion as well (cf. stage *f*, Fig. 3).

Some additional data are given concerning the staining reactions of nucleus and chromosomes.

The cytoplasm of the spindle poles is intensely PAS-positive (cf. Fig. 5). The maximum reaction occurs in the prophase. Then the intensity decreases gradually (cf. Figs. 7, 9, 10) and finally the reaction disappears in the telophase.

The nuclear substances give a very weak PAS reaction in prophase. In meta- and early anaphase the chromosomes are PAS-positive; conversely they are PAS-negative in late ana- and in telophase.

The Hale reaction is occasionally faintly positive in prophase but always distinctly positive in meta- and anaphase (cf. Figs. 10 and 11). The telophase chromosomes are Hale-negative.

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THE EFFECT OF SOME INHIBITORS OF OXIDATIVE PHOSPHORYLATION ON THE HISTOCHEMICALLY DEMONSTRABLE PHOSPHATASES

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Although the distribution of phosphatases is relatively well known thanks to cyto- and histochemical methods, it is not yet clear which part these enzymes play in different metabolic processes. This equally holds for phosphomonoesterases as well as for specific enzymes, e.g. ATP-ase, the histochemical detection of which was recently reported [7, 8].

It is particularly interesting to note the role of enzymes in the processes of phosphorylation associated with tissue oxidation. By using specific inhibitors of oxidative phosphorylation it is possible indirectly to find the enzymatic systems involved in this process. Biological and biochemical experiments have demonstrated that 2,4-dinitrophenol and some histological dyes (e.g. Janus Green B) uncouple the oxidative phosphorylation in the cells of animals [4, 9]. A very interesting fact is that these substances at the same time strongly activate mitochondrial and soluble ATP-ase, while the activation of alkaline and acid phosphatase and 5-nucleotidase is insignificant [3, 4].

The effect of inhibitors of oxidative phosphorylation on the activity and intracellular localisation of histochemically detectable phosphatases has seemingly not yet been examined. It is therefore the purpose of the present work to undertake such an examination. We were particularly interested in the possibility of studying the mitochondrial ATP-ase, which in normal cells is latent and very difficult to detect.

MATERIAL AND METHODS

The method of experimentation used is based on that presented ty Dianzani and Scuro [4]. White, 9 months old male rats from our stock colony were used weighing 226–256 g; they were divided into three groups each with 5 animals. They were fed a standard diet, water was given *ad libitum*. Control group no. 1 received one daily intraperitoneal injection of 1 ml 0.9 per cent NaCl for three consecutive days

oup no. 2 received for three days a daily intraperitoneal injection of 2,4-dinitro-enol (DNP in the amount 2 mg in one ml 0.9 per cent NaCl. Group no. 3 received e same doses of Janus Green B (JG). Twenty-four hours after the last injection e animals were killed through decapitation. Subsequent to killing the liver was ken out and frozen in a temperature of dry ice (approx. −70°C) and was then cut Linderstrøm–Lang's cryostat to 10 μ thick sections. After quick drying in exsiccator er P_2O_5, the sections were incubated for two hours at 37°C in one of the mediums ted below (Table I).

TABLE I

Investigated phosphatases and the composition of incubating mediums.

ne enzyme examined and method used	Concentration of phosphatase ester	Buffer or buffer subst.	$CaCl_2$	Pb salts	$MgCl_2$	pH
nosphomonoesterase I (kaline posphatase) eth. Gomori*	Sodium glycerophosphate 0.01 *M*	Sodium barbital 0.04–0.05 *M*	0.1 *M*	—	0.005 *M*	9.2–9.4
nosphomonoesterase II cid phosphatase) eth. Gomori*	—	0.05 *M* acetate buffer pH 5.0	—	$Pb(NO_3)_2$ 0.004 *M*	—	5.0
TP-ase meth. Naidoo & Pratt**	ATP (Na salt) 0.002 *M*	Sodium succinate 0.05 *M*	0.001 *M*	CH_3COOPb 0.0005 *M*	0.002 *M*	6.5
nucleotidase meth. omori*	Adenylic acid from muscles 0.002 *M*	0.1 *M* barbital buffer pH 8.5	0.08 *M*	—	0.005 *M*	8.3 8.5

* Gomori G., Microscopic Histochemistry. University of Chicago, 1953.
** Naidoo, D. and Pratt, O. E., *Biochem. J.* **62**, 465 (1956).

For the detection of ATP-ase the incubating medium used contained as activators oth $MgCl_2$ and $CaCl_2$.

Control sections were incubated in the same manner after previous inactivation f enzymes in the 1 *N* HCl for 30 min.

RESULTS

Alkaline phosphatase.—In control animals only very weak activity of the lkaline phosphatase can be demonstrated in the liver tissue. However, the ctivity of phosphatase is distinct in minute blood vessels within the inter-obular connective tissue. The liver cells yield a weak reaction in the nuclei

and cellular membrane, probably resulting from diffusion, in the vicinity of the blood vessels (Fig. 1).

After the administration of DNP some venous vessels become rather dilated and with a strong reaction. The liver cells in the vicinity of these vessels show a weak activity of the phosphatase in cytoplasm, nuclei and some bile canaliculi (Fig. 2). This phenomenon may, as in control specimens, be interpreted as a diffusion effect.

Under the influence of JG the activity of alkaline phosphatase was distinctly reinforced in cytoplasm, bile canaliculi and in the nuclei. The appearance around the nuclei of a bright zone of cytoplasm not revealing enzymatic activity is characteristic (Fig. 3). The reaction in the cytoplasm is equally diffusible (probably microsomal phosphatase) and granular (probably mitochondrial). The sinusoids in the experimental animals are dilated. This is probably connected with the symptoms of peritonitis observed in all animals receiving JG.

5-nucleotidase.—In the group of control animals the reaction to 5-nucleotidase is stronger than to alkaline phosphatase (Fig. 4). The enzyme is localized in the cytoplasm, in bile canaliculi and in the nuclei of the liver cells. Besides a strong reaction appears in the walls of the blood vessels in the interlobular connective tissue and in the central vein. The reaction in the cytoplasm is diffuse, probably indicating the presence of the enzyme in the microsomes, or granular which probably corresponds to its presence in mitochondria (Fig. 7).

In experimental animals which received DNP the intensity of the reaction is generally like that observed in control animals. At most there is an insignificantly increased reaction in the cytoplasmic granules (probably mitochondria) with the simultaneous weakening of the diffuse reaction (Fig. 5). Generally the strongest reaction appears in the vicinity of the central vein, whereas it is weaker in the periphery of the lobule.

Under the influence of JG the reaction for 5-nucleotidase is variable, in some places strong and in some distinctly diminished. The strongest activity

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All photographs present liver tissue of rats, unfixed, frozen and cut in cryostat in sections 10 μ thick. The histochemical reactions were performed according to the particulars listed in Table I. Figs. 1–6, ×400; Figs. 7–13, ×1200.

Fig. 1. Reaction to alkaline phosphatase in the liver of control rat. Strong reaction in the blood vessels and weak diffusion reaction in the liver cells in the vicinity.

Fig. 2. Reaction to alkaline phosphatase after administration of DNP. Similarly to control, animals show strong reaction in the blood vessels and very weak reaction in the surrounding liver cells.

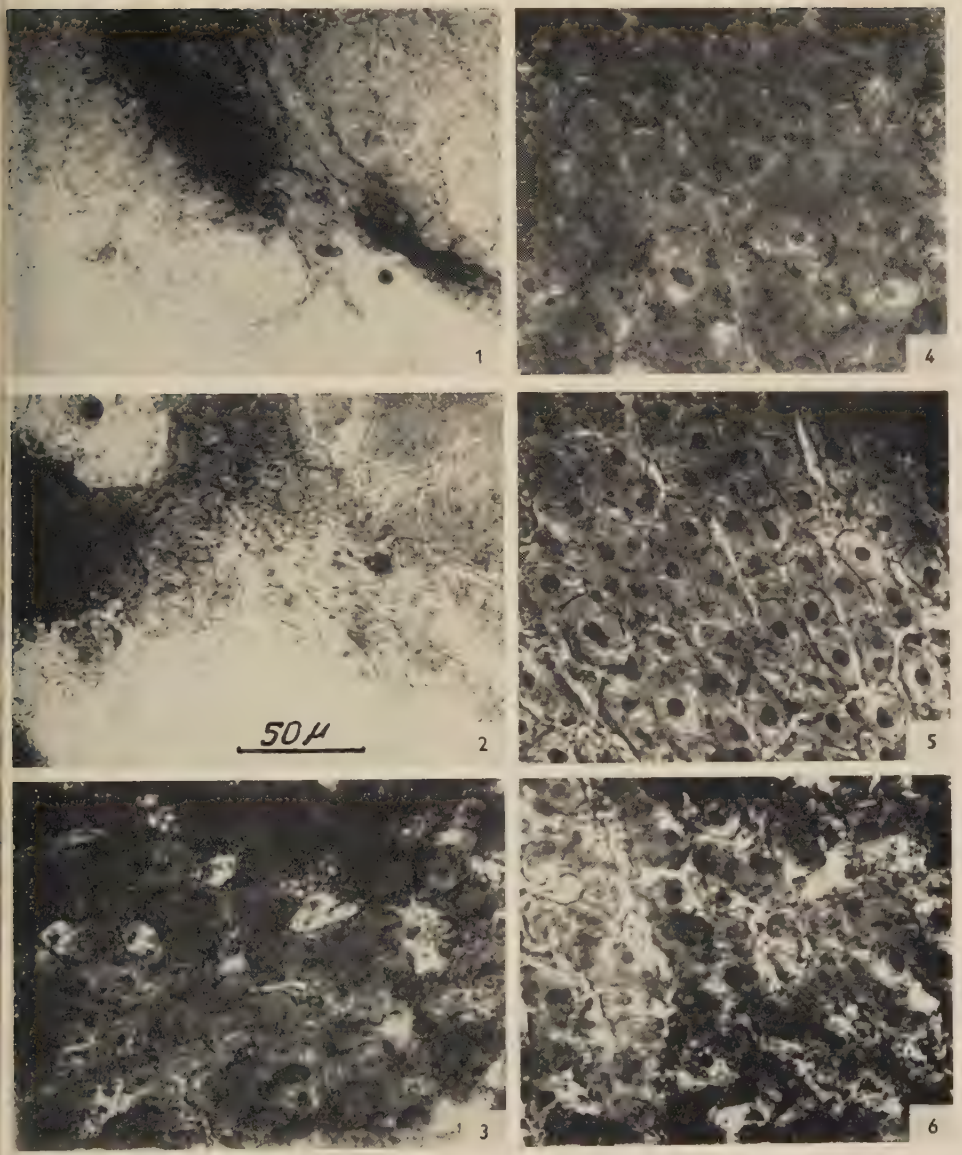
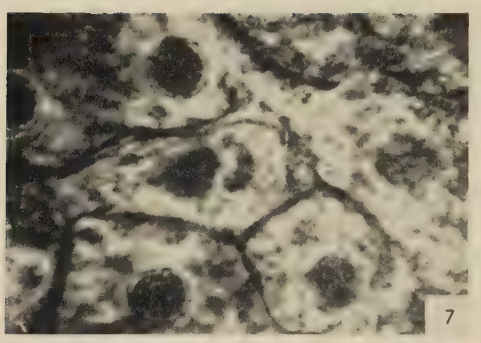
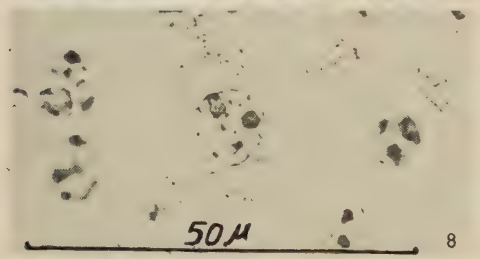
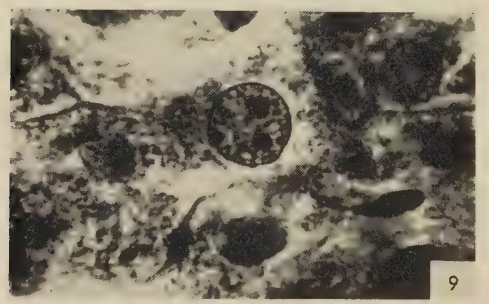
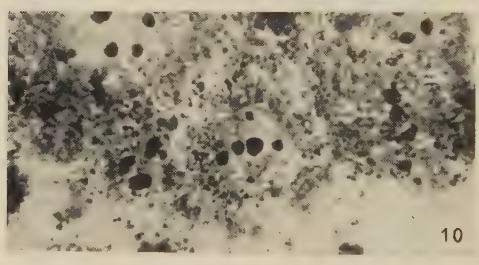


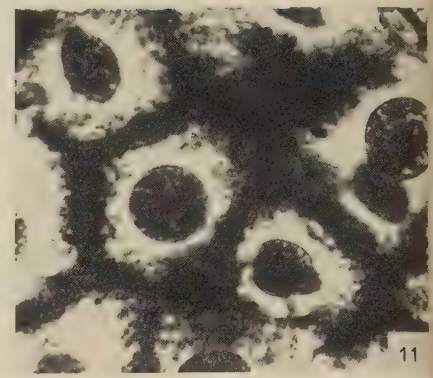
Fig. 3. Alkaline phosphatase after administration of JG. Strong increase in activity of phosphatase in cytoplasm and in the nuclei. Around the nuclei characteristic zones with no reaction.
Fig. 4. 5-nucleotidase in the liver of control rat.
Fig. 5. 5-nucleotidase after administration of DNP. Slight decrease of diffusible reaction, increase of reaction in cytoplasmic granules.
Fig. 6. 5-nucleotidase in the liver of rat after administration of JG. The reaction is ununiform, in some places stronger and in some weaker. The sinusoids are dilated.

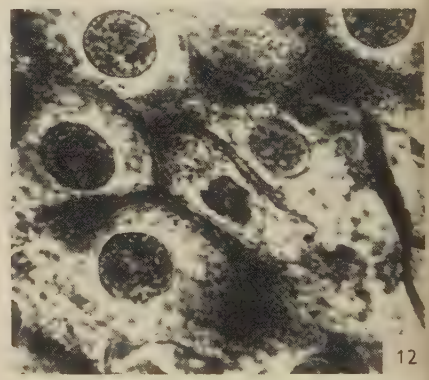












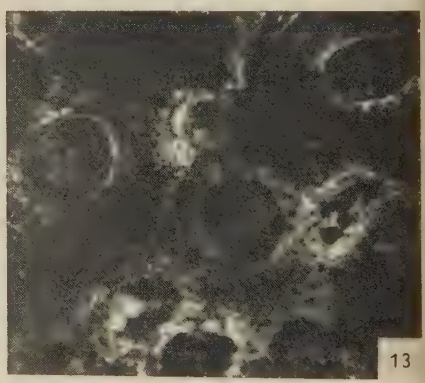


Fig. 7. 5-nucleotidase in the liver cells of control rat.
Fig. 8. ATP-ase in the liver of control rat. The activity of the enzyme is distinctly visible in the nucleoli and weak in nuclear membrane and cytoplasmic granules.
Fig. 9. ATP-ase after administration of DNP. Marked increase of activity in the cellular membrane, in mitochondria, in nuclei and in nucleoli.
Fig. 10. ATP-ase after the action of JG. Increased reaction in nucleoli. Strong reaction in mitochondria. Complete lack of reaction in nuclear and cellular membrane.

pears in the cells lying in the central parts of the lobule, while the farther m the central vein, the weaker is the activity of 5-nucleotidase in the cells. rticularly weak is the diffuse reaction in the cytoplasm pointing to a mage of the soluble or microsomal enzyme. A more distinct activity is eserved in the cytoplasmic granules, probably mitochondria, and the clei (Fig. 6). The blood vessels, venous and arterial, are dilated, but the tivity of 5-nucleotidase is well preserved in them.

Adenosine triphosphatase (ATP-ase).—In our preliminary research we have ed incubating media containing $CaCl_2$, or $MgCl_2$ or both as activators. In nformity with the chemical analyses carried out by Dianzani and Scuro isolated mitochondria, the strongest histochemical reaction for the mito- ondrial ATP-ase was found after incubation in a medium containing both aCl_2 and $MgCl_2$.

In the liver cells of control animals the reaction for ATP-ase is weak and sible mainly in the nucleoli. Moreover the enzyme is present in the mem- ane of the nuclei and in the granules inside the nuclei. Sometimes the sitive reaction is given by a few cytoplasmic granules which according to eir appearance, correspond to mitochondria (Fig. 8).

Under the influence of DNP the reaction for ATP-ase is markedly increased. distinct positive reaction is present in the cellular membrane, in the bile naliculi, in mitochondria and in ground cytoplasm. The latter reaction oints probably to the presence of ATP-ase in microsomes or to the so-called soluble ATP-ase" [4]. A strong activity of ATP-ase is present in the nuclear embrane and probably also in the chromatine (Fig. 9). Particularly a arked activity of ATP-ase was demonstrated in the nucleoli.

In the liver cells of rats receiving JG, the activity of ATP-ase is strongly creased, but the distribution of the enzyme is different from that found in nimals receiving DNP.

A characteristic feature is the complete lack of reaction in the cellular embrane, in bile canaliculi and in the nuclear membrane. A positive reac- on is present exclusively in nucleoli and in the numerous, spheric granules f the cytoplasm (probably mitochondria) densely grouped around the

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ig. 11. Acid phosphatase in control rat. Clear reaction in the nuclei, in the cellular boundaries nd in peripheric cytoplasmic granules. Reaction in nucleoli is invisible.

ig. 12. Acid phosphatase after the action of DNP. Increase of activity in peripheric granules cytoplasm.

ig. 13. Acid phosphatase after the action of JG. Marked increase of reaction in cytoplasm and the nuclei. Around the nuclei the characteristic bright zones with no reaction.

nuclei but also scattered in the whole cytoplasm (Fig. 10). The boundaries of the cells and nuclei are completely invisible.

Acid phosphatase.—In liver cells of control animals the reaction for acid phosphatase is very distinct, above all in the nuclei and the membrane of parenchyma cells and in the walls of bile canaliculi (Fig. 11). The cytoplasmic granules grouped at the periphery of the cells also give a positive reaction. In contrast to the results with ATP-ase the reaction for acid phosphatase in the nucleoli is very weak or even negative.

Under the influence of DNP the activity of acid phosphatase is slightly increased in the cytoplasmic granules (mitochondria) whereas the activity in the nuclei and in the cellular membrane area remains unchanged (Fig. 12).

In animals receiving JG the activity of the acid phosphatase in liver cells is markedly increased (Fig. 13). In the cytoplasm the reaction is diffuse or granular; the nucleus is surrounded by a narrow perinuclear zone which reveals no activity of the enzyme. This zone is narrower than that appearing at the testing of the alkaline phosphatase activity. The marked increase both of the acid phosphatase and the ATP-ase activity shows that Janus Green and 2,4-dinitrophenol act in different ways on the enzymes examined here (cf. Figs. 9, 12 and 10, 13). The effect on the metabolic processes may therefore also be different.

DISCUSSION

The results obtained in the present histochemical investigations may be collected and discussed as follows.

DNP and JG agents which uncouple the oxidative phosphorylation cause a distinct stimulation of the mitochondrial ATP-ase activity in the liver cells of rats. This is in keeping with the chemical researches of Dianzani and Scuro [4] on isolated mitochondrial fractions. In addition to their observations, our results reveal that DNP and JG are activating not only the cytoplasmic ATP-ase (mitochondrial and diffuse) but also that present in the nuclei and nucleoli. From this it can be inferred that also the nuclear enzymes take part in the oxidative phosphorylation. Apart from the papers of Brachet [1, 2] no detailed data are available on this subject. Further chemical estimations should be carried out on isolated nuclear fractions.

Lindberg and Ernster [6] have considered two different ways of explaining the activation of phosphatase through DNP. Either pre-existing phosphatases may be activated, as some authors assume, or DNP causes a cleavage of transphosphorylases, as other authors have maintained. The fragments of

e transphosphorylase may possess the properties of the phosphatases. If we cept this second possibility, the results presented above indicate that a ansphosphorylase system exists not only in the mitochondria, but also in the ucleus and nucleoli.

It appears from our investigation that the latent mitochondrial ATP-ase n be detected by histochemical methods, but only after the chemical amage of mitochondria and with the use of incubating medium containing oth Mg^{++} and Ca^{++} ions. According to the opinion of Ernster and Löw [5], e Ca^{++} ions are transforming the mitochondrial ATP-ase from inactive into tive form, and Dianzani and Scuro [4] believe that it is due to a damaging tivity of Ca ions on the mitochondrial membrane, by which its permeability ould increase and thus unmask the ATP-ase activity.

The histochemical reactions are pointing to a different mode of action of NP and JG. In animals submitted to the action of JG a complete lack of TP-ase activity prevails in the nuclear membrane, though the histological icture of the liver cells reveals no signs of damage of this membrane. JG in ontrast to DNP induces also the activation of the acid and alkaline phos-hatase. The appearance of a bright perinuclear zone indicates that the ctivity of these phosphomonoesterases is inhibited in the nuclear membrane rea and is a further sign of impairment of this membrane. Possibly, JG cts by causing a damage of the nuclear membrane and the adherent cyto-lasm, which may lead to a certain isolation of nucleus from cytoplasm. his isolation of the nucleus may interrupt the oxidative phosporylation, as cording to Brachet [1, 2] the nucleus takes part in linking the processes of xidation and phosphorylation. The increase of the mitochondrial ATP-ase ctivity may be the result of a similar change caused by the action of JG amage on the mitochondrial structure and particularly on their membrane. his may cause the unmasking of latent ATP-ase and possibly of other phos-hatases.

SUMMARY

1. The effect of inhibitors of oxidative phosphorylation 2,4-dinitrophenol DNP) and Janus Green B (JG) on the activity and distribution of histo-hemically detectable phosphatases in the liver cells of rats was investigated.

2. Unfixed, frozen liver tissue was sectioned in cryostat and then incubated vith the aim of detecting the alkaline phosphatase, acid phosphatase, ATP-se and 5-nucleotidase.

3. Dinitrophenol and Janus Green as well stimulate distinctly the mito-

chondrial and nucleolar ATP-ase activity. DNP also stimulates the activity of ATP-ase in nuclei and in the nuclear and cellular membrane, while JG abolishes completely the activity of the enzyme in these structures.

4. Dinitrophenol stimulates only slightly the activity of acid and alkaline phosphatases and 5-nucleotidase, while Janus Green distinctly stimulates the activity of acid and alkaline phosphatases.

5. Special attention is focused on the possibility of detecting mitochondrial ATP-ase and on the different actions of mechanisms of dinitrophenol and Janus Green.

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THE EFFECT OF ANAEROBIOSIS ON THE ORIGIN OF RESPIRATORY-DEFICIENT YEAST[1]

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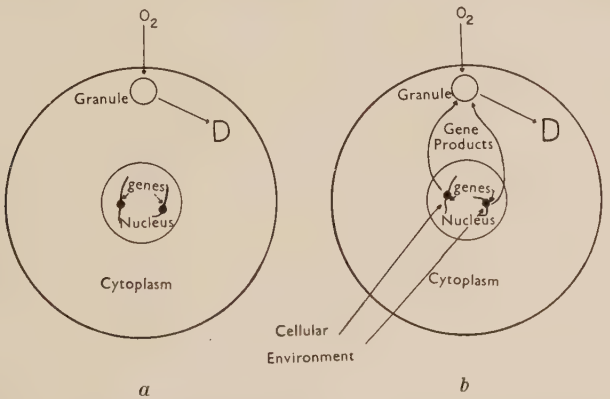
has been obvious for a long time that the problem of cancer involves the oblem of dedifferentiation. The view that differentiation (and dedifferentiation) is probably effected by an extrachromosomal mechanism has its origin the fact that the genome of the soma is generally identical throughout the ferent tissues. Warburg [8] has proposed a specific theory of the origin of ncer assuming that the materials which maintain differentiated tissue egrity are produced by the localized use of oxidative energy at specific es on cytoplasmic granules. Loss or degradation of the granules could sult in dedifferentiation, provided the cell became adapted to anaerobiosis r the energy necessary for survival. Contamination of the surface of the anules by homologues of oxidizable materials could lead to degradation the oxidative apparatus and a consequent loss of tissue specificity. Inactition of the aerobic mechanism by simple anaerobiosis could bring the an-



g. 1. (*a*) Warburg's theory of tissue differentiation. Tissue differentiation is achieved by an idative reaction on the cytoplasmic granule resulting in *D* which differentiates the specific sue. (*b*) Modified theory of tissue differentiation. Adaptive gene-products produced in a specific llular environment are oxidized on the surface of the cytoplasmic granule to produce *D*.

[1] This work was supported by a research grant from the Illinois Division of the American ncer Society.

aerobic mechanism into full expression and lead to degradation of the aerobic mechanism and thus to dedifferentiation. Small colonies arising from respiratory-deficient (aer) cells arise spontaneously in yeasts [5, 2] at a rate that is greatly increased by numerous toxic substances without otherwise impairing cellular metabolism.

EXPERIMENTAL

Culture 8256 of the Carbondale breeding stock is an adenine-deficient, pink culture which produces respiratory-deficient (aer) colonies which are a light-colored, brownish-orange [7] in contrast to the bright pink of the respiratory-sufficient (AER) colonies. Although colony size is an adequate diagnostic criterion in the rapidly growing diploid cultures, it is not adequate when adenine-deficient cultures are used since they grow rather poorly. Size alone is generally a poor criterion since it is carbohydrate-limited; colonies of respiratory-deficient cells grow to the same size as colonies of respiratory-sufficient cells if the medium contains a high concentration of carbohydrate. Since haploid cultures tend to cluster it is difficult to determine the frequency of respiratory-deficient (aer) cells by colony-size alone, for a cluster, containing a single respiratory-sufficient (AER) cell, would produce a large colony. (Diploid and tetraploid cells do not cluster but produce so few aer variants that experiments of this type are not feasible with them.)

TABLE I

Counts of respiratory-deficient and respiratory-sufficient colonies after aerobic and anaerobic incubation in buffer solution.

		Aerobiosis		Anaerobiosis	
Time (days) ...	0	1	2	1	2
No. of plates counted	16	6	4	7	4
Total colonies	2770	1046	635	998	415
aer colonies	46	8	4	13	4
single colonies	25	3	1	8	1
sectored colonies	21	5	3	5	3
Percentage aer	1.66	0.77	0.63	1.30	0.96
single colonies	0.90	0.29	0.16	0.80	0.24
sectored colonies	0.76	0.48	0.47	0.50	0.72
Cells per ml of culture solution total	2.0×10^4	2.3×10^4	3.5×10^4	2.2×10^4	2.3×10^4
aer	3.4×10^2	1.8×10^2	2.2×10^2	2.9×10^2	2.2×10^2

The effect of anaerobiosis on the production of aer cells in the absence of growth.—An periment was performed (Table I) to determine if exposure to anaerobic conditions the absence of growth would suffice to increase the frequency of aer cells in a pulation. Aggregation of the cells in this experiment would not be important since e degree of aggregation would not be expected to change. Suspensions of washed ls of culture 8256 containing about 20,000 cells per ml were exposed to aerobic d anaerobic conditions in fifty ml Erlenmeyer flasks; the flasks were placed in an aerobic jar and nitrogen was passed through the jar for 30 minutes to replace the . About 20 ml of 10 per cent pyrogallol was added to a beaker containing an equal nount of 50 per cent KOH solution (previously placed in the jar) to remove the maining oxygen. The aerobic cultures and the anaerobic jar were shaken in an cubator at 30°C. Samples of cultures were spread on nutrient[1] agar containing 2 per nt sugar at the times indicated. The bright pink and brownish orange colonies and ctors were counted after incubation. Most sectored colonies contained only a single ctor (presumably due to their origin from a pair of cells—an aer bud on a AER other cell). No significant increase or decrease in aer cells was observed either in robiosis or anaerobiosis. Anaerobiosis in the absence of growth does not bring about ss of respiratory ability. A similar result was obtained when sugar alone was added the buffer showing that dedifferentiation does not occur in an actively fermenting ll in the absence of division.

The production of aer cells during growth under anaerobiosis.—Study of the echanism of dedifferentiation of AER to aer is nearly impossible if the aer lls divide significantly more rapidly than the rate at which they originate om AER cells. When standard nutrient medium is made up without carbohydrate, aer cells multiply either very slowly or not at all [6]. A parallel xperiment was performed using nutrient broth (without added carbohydrate) n place of buffer. The colony counts in Table II show a significant increase n the percentage of aer cells under anaerobiosis compared with aerobiosis. he final row in Table II shows that aer cells grow very poorly, if at all, nder similar conditions.

One of the complications in this analysis arises from the fact that haploid ells tend to aggregate and if the colonies of aer cells arose primarily from ingle cells while those of AER cells arose from aggregates, a misleading conlusion might be drawn. Table III reveals the state of aggregation of the cells n the experiment described in Table II. Although aggregation decreases as rowth progresses, the rates are so similar under aerobiosis and anaerobiosis nat the effects may be inferred to have cancelled out. It is possible from these ata to correct the observed frequencies of aer cells as indicated in the last ow in Table III. Anaerobiosis produces a ten-fold increase in the popula-

[1] Peptone 3.5 g, KH_2PO_4 2 g, $MgSO_4$ 1 g, $(NH_4)_2SO_4$ 1 g, liquid yeast extract 3 ml, B-vitamin ixture 1 ml in 1 l water.

TABLE II

Counts of respiratory-deficient and respiratory-sufficient colonies after aerobic and anaerobic growth in nutrient medium without added carbohydrate.

		Aerobiosis		Anaerobiosis	
Time (days) ...	0	1	2	1	2
		Culture: 8256 AER			
No. of plates counted	16	2	12	8	9
Total colonies	2770	999	1298	1430	1360
aer colonies	46	5	9	53	66
single colonies	25	2	3	20	30
sectored colonies	21	3	6	33	36
Percentage aer	1.66	0.50	0.69	3.70	4.85
single colonies	0.90	0.20	0.23	1.40	2.20
sectored colonies	0.76	0.30	0.46	2.30	2.65
Cells per ml of culture solution					
total	2.0×10^4	1.2×10^6	1.9×10^6	4.3×10^5	1.0×10^6
aer	3.4×10^2	6.0×10^3	1.3×10^4	1.6×10^4	4.8×10^4
		Culture: 8256 aer			
Cells per ml of culture solution	2.4×10^3	—	3.7×10^3	—	2.7×10^3

TABLE III

Hemocytometer counts during aerobiosis and anaerobiosis.

	Aerobiosis		Anaerobiosis	
Time (days) ...	1	2	1	2
Total aggregates counted	34	91	53	64
single cells	13	64	15	38
clusters	21	27	38	26
per cent single cells	38.2	70.3	28.3	59.3
No. of cells in total aggregates	ca. 400	ca. 350	ca. 400	ca. 200
Corrected percentage of aer cells	0.50	0.30	5.00	3.80

n of aer cells and, since the latter do not grow in this particular medium, e increase in aer cells must have resulted from dedifferentiation of AER to r.

DISCUSSION

This analysis confirms Warburg's view that growth under anaerobiosis ads to degradation of the aerobic apparatus. Confirmation of Warburg's ew does not exclude the possibility that cancer may arise from other types dedifferentiations since Warburg assumed that tissue integrity was con-olled only by an extrachromosomal mechanism. The possibility that gene utation may effect dedifferentiation has been raised by Beerman's [1] udy of the polytene chromosomes of *Chironomus*. Beerman found that the puffs" in polytene chromosomes originate at single bands. The "puffs" are nerally accepted to indicate that the gene is producing its specific gene oduct. Beerman showed that cells from different tissues exhibit characteris-patterns of "puffing" specific for the tissue. This suggests that different ombinations of genes control tissue specificity and it follows that inactivation any one of the specific genes controlling differentiation of a specific tissue ould lead to dedifferentiation of the cell.

On the basis of Beerman's work, Warburg's concept may be restated as llows: Tissue differentiation is achieved by the adaptive response of genes a specific cellular environment. "Ontogeny may involve a series of (gene-ntrolled) adaptive reactions each of which results in a shift in equilibrium, nditioned by the changed environment which continued multiplication roduces" [3]. The materials which maintain tissue specificity are produced the cytoplasm (only by oxidative metabolism) at specific sites on the cyto-lasmic granule, through the control of gene-products arising from cells hich find themselves in a specific adaptive environment. Dedifferentiation an occur either (*a*) as Warburg proposes by degradation of the cytoplasmic erobic mechanism or (*b*) by destruction of genes which produce the specific aterials the oxidation of which controls the maintenance of tissue specificity.

SUMMARY

Respiratory-sufficient (AER) haploid yeast cells were subjected to aerobic nd anaerobic conditions to determine whether anaerobiosis transforms re-piratory-sufficient cells into respiratory-deficient (aer) cells. Anaerobiosis in ne absence of growth failed to increase the frequency of aer cells. Growth of ER cells under anaerobiosis resulted in a 10-fold increase in aer variants

over the frequency under aerobiosis. Control experiments showed that the increase was not due to the dispersal of cell aggregates or the selection of aer cells. These experiments confirm Warburg's hypothesis that anaerobiosis may lead to the degradation of the oxidative mechanism. It is proposed that dedifferentiation which might lead to cancer can occur either by the degradation of the cytoplasmic aerobic mechanism or by destruction of genes which produce the specific materials, the oxidation of which controls the maintenance of tissue specificity.

We are pleased to acknowledge the assistance of Mr. David D. Pittman and Dr. Maurice Ogur, who have made many helpful and valuable suggestions.

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SUSPENSION CULTURES OF STRAIN L MOUSE FIBROBLASTS

I. A GLASS STIRRER APPARATUS FOR THE CULTIVATION OF CELL SUSPENSIONS[1]

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:VERAL investigators [3, 5, 6, 8, 9] have reported methods for growing sue cells in suspension cultures. However, all the methods have one com- on drawback, that the environmental conditions cannot be kept even rela- ely constant throughout the culture period.

The purpose of this paper is to describe a glass stirrer apparatus, a modi- ation of the model described by Evans, Earle, Sanford, Shannon and Waltz], in which cells can be grown in suspension under known, controlled nditions.

Evans and co-workers [7] designed a glass stirrer apparatus for the prepara- on of replicate samples of a cell suspension. In the development of this chnique they found that stirring suspensions of the strain L mouse fibro- ast at a speed of 950 rpm for two hours did not influence subsequent growth the cells on perforated cellophane in T-12 flasks. Such evidence suggested at this stirring apparatus might be adapted for use as a culture vessel for owing cells in suspension.

MATERIALS AND METHODS

Design of apparatus.—The modified glass stirring apparatus (Fig. 1) has the same sic design as that used by Evans and collaborators [7]. However, some basic odifications have been made to make the apparatus more useable as a culture essel.

[1] The initial phase of this investigation was aided by a U.S. Public Health grant administered Dr. P. J. Leinfelder, Department of Ophthalmology, State University of Iowa. The main part the research was supported by a grant from the American Cancer Society on the recommenda- on of the Committee on Growth of the National Research Council.

[2] On leave of absence from the Departments of Ophthalmology and Physiology, College of edicine, State University of Iowa, Iowa City, Iowa, U.S.A.

The capacity of the reservoir has been increased from 70 to 250 ml. The design and dimensions of the glass stirrer have not been altered. Therefore, the clearance between the stirrer blade and the reservoir wall has been increased from 4 to 12 mm.

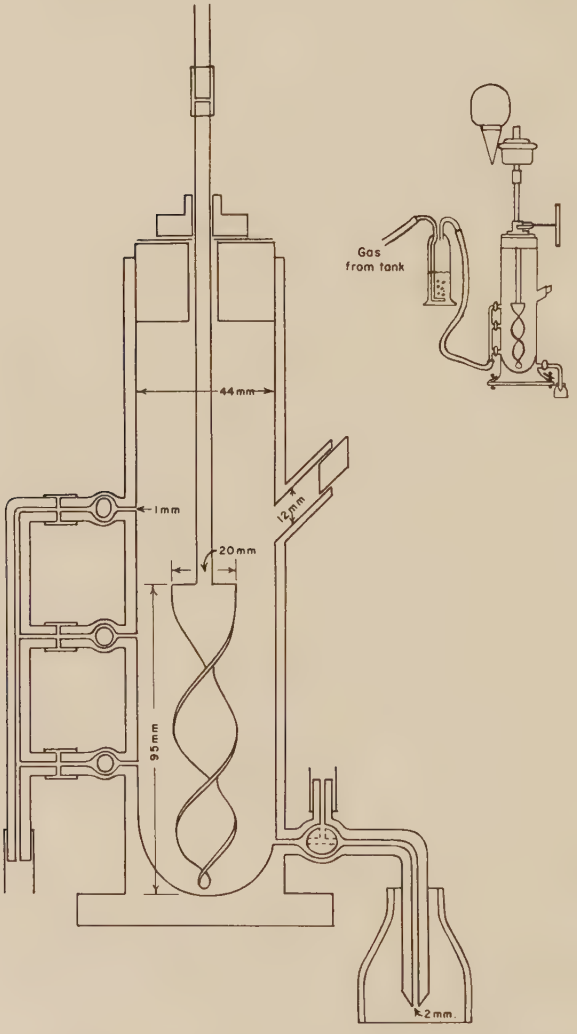

Fig. 1. Glass stirring apparatus for the cultivation of cells in suspension (adaptation of the stirrer apparatus of Evans *et al.* [7]).

In order to insure adequate gassing of the culture, a gassing manifold has been incorporated into the side of the reservoir so that the culture can be gassed at different levels depending upon the level of the fluid.

The side arm was used for the addition of nutrient fluid.

The chief technical difficulty was the accumulation of cells at the fluid-air interface

s was avoided by a combination of three factors: (1) The inner glass surface of reservoir and the glass stirrer were siliconed. (2) Any vibration of the stirrer was vented (*a*) by replacing the teflon cap used in the Evans *et al.* [7] stirrer with a ber stopper and (*b*) by supporting the stirrer in a clamp just above the stopper. Instead of being held in a clamp stand, the reservoir sat on the floor of the incubator. In addition the reservoir was bolted to the incubator floor to insure stability. en all three precautions were taken, the meniscus remained very sharp and the s did not accumulate at the fluid-air interface.

A variable speed cone drive motor[1] was used as the stirring motor.

Preparation of cell suspension.—Strain L, No. 929, mouse fibroblasts were grown T-60 flasks (5) on glass in 15 ml of 40 per cent horse serum, 20 per cent (1:1) chick bryo extract and 40 per cent Tyrode's solution [2]. Cultures were grown for three ys so that the parent culture had a cell population of approximately ten million. In order to remove the cells from the glass floor of the flasks, the supernatant fluid s removed and the cells were treated with an 0.5 per cent trypsin[2] solution for a iod of five minutes at room temperature.[3] The cell suspension was then centrifuged 1000 rpm for five minutes. The trypsin solution was decanted and the cells were uspended in Tyrode's solution which had been previously warmed to approxitely 37°C. After a second centrifugation, the Tyrode's solution was decanted and e cells were resuspended in 20 ml of nutrient fluid.

The nutrient fluid consisted of 45 per cent horse serum, 10 per cent (1:1) embryonic tract and 45 per cent Tyrode's solution, and contained per ml 500 units penicillin d 0.05 mg streptomycin. Equal parts of cell conditioned fluid, nutrient in which e cells had been grown in the T-60 flask, and new fluid were combined to make the tial suspending fluid.

The cell suspension was then put through a platinum sieve [7] to remove any bris.

At this time a sample of the suspension was introduced into a haemacytometer amber and the cell population determined. The cell concentration was then adsted to approximately 500,000 cells per ml by the addition of suspending fluid.

Twenty ml of this cell suspension was then introduced into the stirring apparatus ich had previously been assembled in the incubator whose temperature was 37.5°C.

Stirring and gassing of cell suspension.—The cell suspension was stirred continusly at a speed of 230 rpm. In preliminary experiments 115 rpm was also used. The culture was gassed continuously with a slow flow of 95 per cent air and 5 per nt carbon dioxide. The gas was first passed through a cotton packed bacterial ter. This filter was changed every 24 hours to insure effective sterilization of the s. Then the gas mixture was saturated with water vapor at 37.5°C by passage rough a water bottle in the incubator.

Counting procedure.—A sample of cell suspension (approximately 100 microliters) as taken by use of a Pasteur pipette. Duplicate counts were made in an A.O. ight-line haemacytometer.

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[1] Sargent cone drive stirring motor, Cat. No. 76445.

[2] Crystalline trypsin preparation prepared by the Novo Terapeutisk Laboratorium A/S, penhagen, Denmark.

[3] The cells could have been freed from the glass by shaking the flask. However, trypsin treatent was used to obtain a suspension containing only single cells.

Table I. Determination of the accuracy of the counting procedure and the uniformity of the cell suspension by cell counts at different levels and regions of the culture.

	ORDER SAMPLES TAKEN	NUMBER OF CELLS COUNTED IN 9 cu.mm.		CELLS PER ML. (Thousands)	
			Mean of duplicate samples		Mean of duplicate samples
	3	320 287	303.5	355.6 318.9	337.3
	5	287 315	301.0	318.9 350.0	334.5
	2	277 268	272.5	307.8 298.9	303.4
	4	309 283	296.0	343.3 314.4	328.9
	6	295 277	286.0	327.8 307.8	317.8
	1	305 309	307.0	338.9 343.3	341.1
	7	273 295	284.0	303.3 327.8	315.6
	± Mean st. error	292.9 ± 16.6	292.9 ± 11.7	325.5 ± 18.4	325.5 ± 13.0
	± St. error in %	5.7	4.0		

Additional statistical analysis:

The variance on single countings estimated from double countings was 267 with 7 degrees of freedom.

The variance on single countings estimated from the means was 287 with 6 degrees of freedom.

Fischer's z test comparing these two variances showed no significance.

The total estimate of the variance on single countings was 276 with 13 degrees of freedom.

The accuracy of the counting procedure was evaluated statistically (Table I). When 300 cells per sample were counted, the standard error was 5.7 per cent.

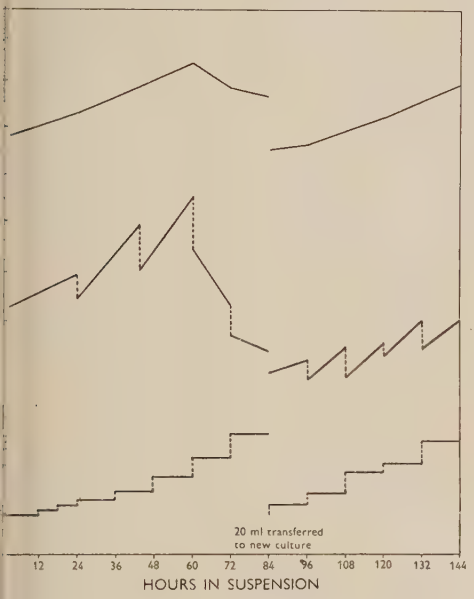
In order to study cell clumping, a differential count for clump formation was routinely done. Cell clumps consisting of 2 to 8 cells were counted separately. Whereas larger cell clumps were counted as clumps of 10 cells as the number of cells per clump could not be accurately determined.

Renewal of culture fluid.—Every 12 hours the cell concentration was determined. At this time 25 per cent new fluid was always added. In most experiments this addition of new fluid reestablished the cell concentration at approximately 500,000 cells per ml. If additional fluid was needed to reduce the cell population to this level, cell conditioned fluid was used.

RESULTS

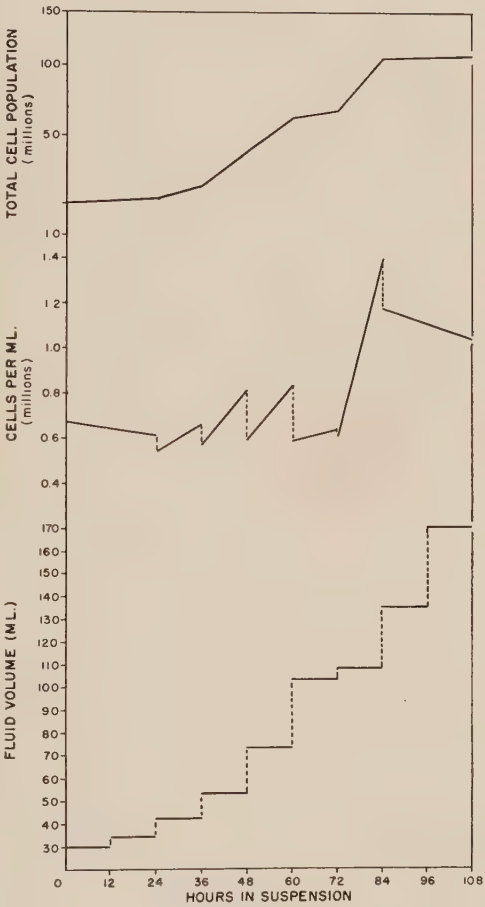
Activity under Controlled Conditions

The growth curve.—Approximately logarithmic growth was maintained if the cell concentration did not exceed 1,300,000 cells per ml and (2) 25 per cent new fluid was added every 12 hours (Figs. 2 and 3).



g. 2 (*left*). Suspension culture grown under atively constant environmental conditions. tial suspending nutrient composed of equal rts fresh and cell conditioned fluid. 25 per nt fresh fluid added every 12 hours.

g. 3 (*right*). Suspension culture in which cell ncentration was controlled until 72 hours by addition of varying quantities of new fluid ery 12 hours. Fresh fluid was used as the initial spending medium.



Under these environmental conditions the generation time, that time cessary to double the cell population in the logarithmic growth phase, eraged 29.8 ± 2.7 hours. The mitotic coefficient, the per cent of dividing lls per 1000 cells, was found to be approximately 2.2.

Uniformity of the cell suspension.—During the logarithmic growth phase over 90 per cent of the cells were single or double cells (Fig. 5). Clumping did not occur under controlled culture conditions.

In order to determine if there was a uniformity of cell distribution throughout the culture, cell samples were taken at different levels and regions of a culture. As shown in Table I the distribution of cells was uniform. Cells did not tend to settle out at the periphery of the reservoir or did they tend to settle to the bottom.

Morphology.—Cells grown in suspension for prolonged periods appeared morphologically very healthy (Fig. 4). Excessive fat droplets and vacuoles were not seen in these cells. The cells tended to become more uniform in size after a few days in suspension.

When these cells were reintroduced into a T-60 flask, they adhered quite quickly, within an hour, to the glass floor.

Activity under Varying Environmental Conditions

The influence on growth of varying different environmental conditions was studied initially in order to establish the standard controlled culture conditions.

Speed of stirring.—The standard speed of stirring was 230 rpm. To determine the minimum required rate of stirring, 115 rpm was used. As shown in Fig. 5, cell clumping definitely increased with reduced speed of stirring.

Addition of New Fluid and the Cell Fluid Ratio

As shown in Fig. 6 the addition of 20 per cent or less of new fluid every 24 hours was insufficient to support logarithmic growth. Growth gradually decreased until complete cessation.

The addition of 25 per cent new fluid every 12 hours supported logarithmic growth if the cell population was kept between 400,000 and 1,300,000 cells per ml (Figs. 2 and 3). As seen in both experiments, Figs. 2 and 3, growth fell off when the cell concentration exceeded 1,300,000 cells per ml.

Cell conditioned medium.—When the cells were initially suspended in completely fresh fluid, there was a lag phase of approximately 24 to 36 hours

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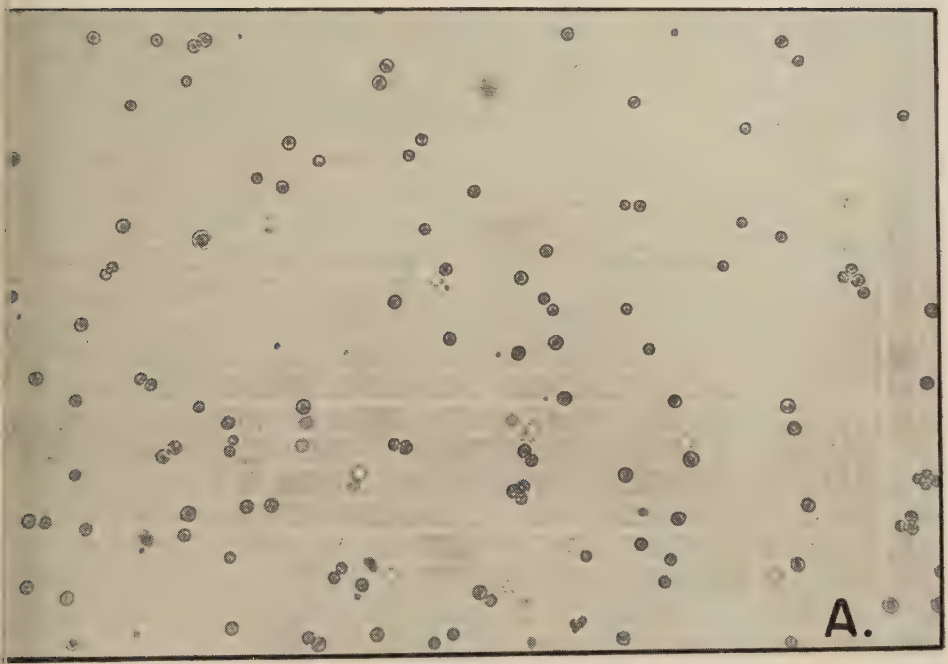
Fig. 4. Strain L, No. 929, mouse fibroblasts from the suspension culture Fig. 2 after 144 hours in suspension. *A*, Living cells photographed in an A.O. bright-line haemacytometer. × 75. Cells are not compressed since the chamber is 100 microns deep. *B*, Living cells photographed under oil immersion. × 1080. *C*, Cells stained with Eskelund's sex chromatin stain (unpublished). × 1080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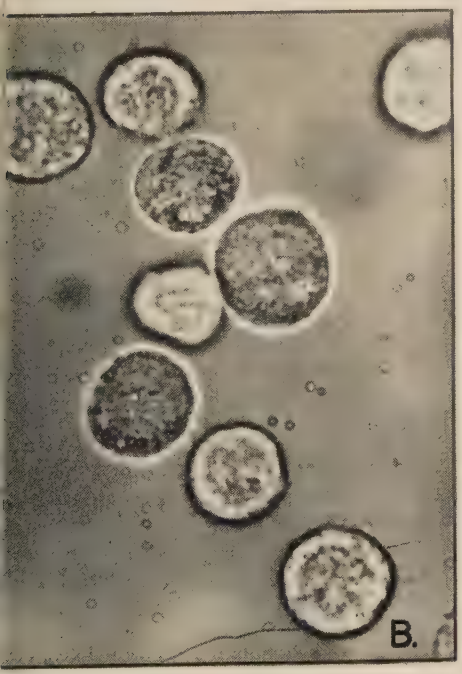
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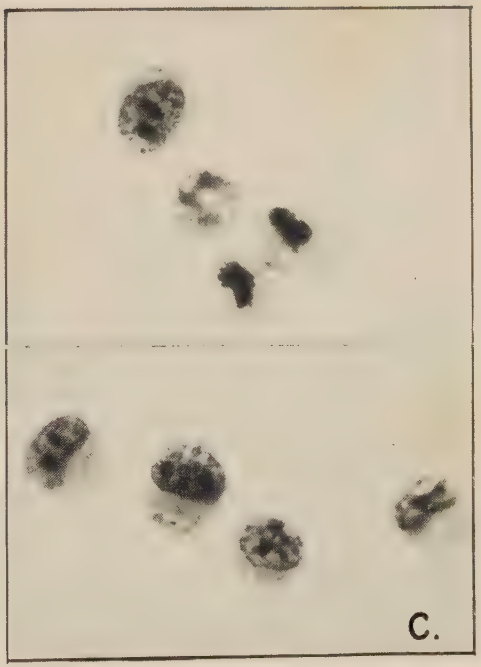

C.

(Figs. 3 and 6). However, if the suspending fluid contained 50 per cent cell conditioned fluid already during the initial 12 hours logarithmic growth occurred and the initial lag phase was eliminated (Fig. 2).

There was some evidence that the addition of more then 35 per cent new fluid at 12 hour intervals depressed growth (Fig. 3). Therefore, conditioned

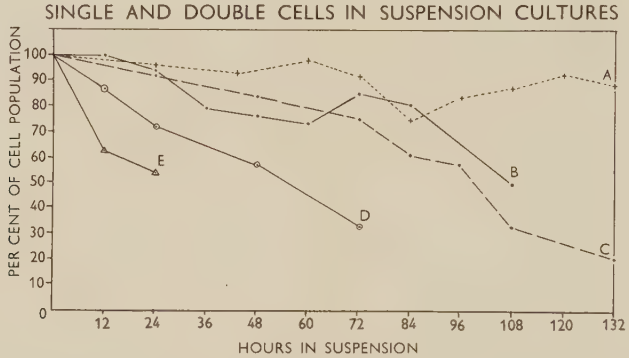


Fig. 5. The percentage of single and double cells in suspensions under various culture conditions. *A*, Exp. Fig. 2. Constant envir. conditions: Cell concentration kept below 1,300,000 cells per ml (except at 60 hrs.) by the addition of 25 per cent new fluid every 12 hrs. *B*, Exp. Fig. 3. Cell concentration controlled (700,000 ± 150,000 cells per ml) until 72 hrs. by the addition of varying quantities of new fluid (20–41 per cent) every 12 hrs. *C*, Exp. Fig. 6. Varying environmental conditions: Cell concentration increased to 2,000,000 cells per ml by 132 hrs. as only 20 per cent or less new fluid was added every 24 hrs. *D*, Reduced speed of stirring—115 rpm. *E*, Deficient nutrient fluid—the medium contained no embryonic extract.

fluid was used if more than 25 per cent dilution was required to reduce the cell concentration.

Cells grown under the above varying environmental conditions tended to form clumps (Fig. 5).

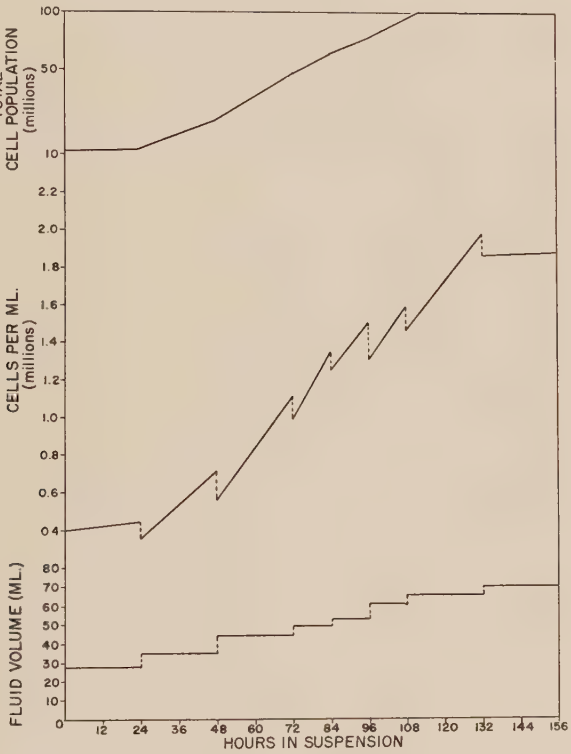
DISCUSSION

The glass stirring apparatus of Evans, Earle, Sanford, Shannon and Waltz [7] was successfully adapted for use as a culture vessel for growing cells, at least the strain L mouse fibroblast, in suspension under known, relatively controlled conditions.

Increasing the clearance of the stirrer blade from 4 mm to 12 mm did not cause the cells to settle out peripherally or influence the distribution of cells throughout the column of suspension (Table I). This modification simplified the assembling of the apparatus.

The greatest difficulty in adapting the glass stirrer apparatus was the prevention of the accumulation of cells at the fluid-air interface. This pro-

ced a ring of cell sediment in the zone of oscillation of the meniscus. The s of cells to this ring exactly balanced the increase in cell population during 24-hour period. This problem was completely solved by siliconing the ner glass surface of the reservoir and the stirrer blade and eliminating any ration in the apparatus.



g. 6. Suspension culture grown der varying environmental nditions. Initial suspending id was completely fresh fluid. per cent or less new fluid s added every 24 hours.

The minimum speed of stirring found to be sufficient for growing the rain L fibroblasts in suspension was 230 rpm. This finding is in agreement ith the work of Earle and his collaborators [6] who found that such a speed as optimum for the growth of fixed tissue cells in fluid suspensions con-nuously agitated on a Brunswick shaker.

The suspension culture was gassed continuously with a slow flow of umidified 95 per cent air and 5 per cent carbon dioxide. Two factors were ken into consideration in determination of the rate of flow: (1) The total ll population rarely exceeded 100,000,000 cells. (2) On the basis of a res-iration of 2×10^{-5} microliters per hour per cell for the 7-day embryonic

chick heart fibroblast [4] the maximum amount of air required for efficient aerobic metabolism would have been 10 ml per hour. It was assumed that any flow exceeding this volume, though not constant, would be adequate.

Several technical difficulties encountered in other methods for growing cells in suspension did not occur. No frothing or adherence of the cells to the glass wall or stirrer occurred. Proteinogenous precipitate did not form in the nutrient fluid as was noted in tumble tube cultures [9] and occasionally in flask suspension cultures [6].

Approximately logarithmic growth was established initially if the suspending medium was composed of equal parts of cell conditioned and fresh nutrient. Such growth was maintained if a limited, not more than 35 per cent, new fluid was added at 12 hour intervals. These findings confirmed the work of de Bruyn [1] and Owens, Gey and Gey [9] who reported that the addition of 50 per cent cell conditioned fluid enhanced growth. The significance of such findings should receive further consideration.

The main advantage of this culture method was that the environmental conditions might be kept relatively constant throughout the culture period. Both the addition of a constant percentage of new fluid and the maintenance of a relatively constant cell concentration appeared to be mandatory for maintaining the cells in logarithmic growth phase (Figs. 2, 3 and 6). It was not sufficient to add enough fresh fluid to keep the cell concentration below 1,300,000 cells per ml. If less than 25 per cent fresh fluid was added every 12 hours, a logarithmic growth phase was not established (Fig. 6). Fluid addition in excess of 35 per cent appeared to depress growth (Fig. 3). However, if the cell concentration exceeded this level, logarithmic growth was not maintained even though 25 per cent fresh fluid was added (Figs. 2 and 3). If both factors were regulated, growth was approximately logarithmic.

Earle and co-workers [6] reported that suspension of the strain L mouse fibroblast grown in flask cultures continuously agitated on a Brunswick shaker doubled their populations every $2\frac{1}{2}$ to 5 days. As the cells were resuspended in entirely new fluid every $2\frac{1}{2}$ days, logarithmic growth was probably not maintained throughout the entire culture period. Therefore, the shortened generation time, 29.8 ± 2.7 hours, of cells grown in the stirring apparatus was most likely due to more constant culture conditions.

A difficulty encountered in growing fixed cells in suspension has been that the cells tended to form in clumps [6, 9]. In this study it was found that cell clumping appeared to be an indication of adverse environmental conditions (Fig. 5). Such factors as reduction in speed of stirring, alteration in cell fluid ratio, insufficient addition of new fluid, and a deficient nutrient all

used clumping of the cells. Therefore, the impossibility of maintaining latively constant culture conditions may have been the cause for the currence of cell clumping in suspensions grown by other methods [6, 9].

The stirring apparatus was developed for growing cells in suspension under latively constant environmental conditions for short culture periods, not ore than a week. The aim was to obtain suspensions of cells in the logarithc growth phase for metabolic studies. However, the reservoir stirrer paratus could be easily enlarged to allow longer cultivation times.

SUMMARY

The glass stirring apparatus designed by Evans, Earle, Sanford, Shannon d Waltz [7] for the preparation of replicate samples of a cell suspension s been adapted for use as a culture vessel for growing cells in suspension der known, relatively controlled conditions.

Logarithmic growth was maintained if (1) the cell concentration did not ceed 1,300,000 cells per ml and (2) 25 per cent new nutrient fluid was ded every 12 hours. Under these environmental conditions the generation ne averaged 29.8 ± 2.7 hours. The mitotic coefficient was approximately 2.2.

During the logarithmic growth phase over 90 per cent of the cells were ngle or double cells. It was found that cells clumped under varying and lverse culture conditions.

Evidence was obtained to indicate the importance of cell conditioned fluid.

I am much indebted to Dr. Wilton Earle and Dr. Virginia Evans of the National ncer Institute for discussions and for supplying the stock flask of strain L mouse broblasts, and to Dr. Jørgen Kieler for valuable suggestions and for making the cilities of his laboratory available to me.

I also wish to thank Professor Heinz Holter for his advice and encouragement, r. Otto Scherbaum and Mr. Howard Webster.

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BRIEF NOTES

IN VITRO INVESTIGATION OF THE CILIATED AND SECRETORY CELLS IN THE RABBIT FALLOPIAN TUBE

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As there is still much difference of opinion as to whether ciliated cells may be transformed into secretory cells or vice versa in epithelium of the rabbit Fallopian tube [1, 4, 5, 9 and others], the present work has been carried out to investigate (1) whether ciliated and secretory cells appear in epithelial membranes grown from explants of epithelium of the rabbit Fallopian tube in tissue culture, (2) whether transformation of ciliated cells into secretory cells or vice versa occurs *in vitro*, and (3) whether cells showing both ciliary and secretory activity occur in the epithelial membranes.

Method of Culture

Fragments of the mucosa of the rabbit Fallopian tube were cultured by the single coverslip technique using a fluid medium consisting of equal parts of homologous serum and Parker's solution. The coverslips on which particularly large and beautiful epithelial membranes had grown were transferred to special chambers after two or three days' incubation for further study.

Results

The following three types of cell were identified in the epithelial outgrowths: (1) cells showing the characteristics of undifferentiated cells usually appearing *in vitro*, (2) ciliated cells, (3) cells containing specific granules of varying size.

Undifferentiated cells.—Undifferentiated cells were found to predominate. Their structure was identical with that of undifferentiated cells usually appearing *in vitro*. Signs of cilia formation were not identified. Examination of stained preparations revealed that the majority of the protoplasmic inclusions contained lipids. P.A.S.-positive structures were not seen.

Ciliated cells.—Cilia were seen scattered on the surface of the cells. Their behaviour resembled that of the cilia of the cells appearing in cultures of nasal epithelium [6, 8].

Examination of stained preparations showed that the majority of the granules in the cells contained lipid elements. P.A.S.-positive structures were seen within the cytoplasm; these were non-resistant to diastase, irregularly outlined and varied in size. However, phase contrast microscopy of unfixed and unstained epithelial cells failed to reveal them.

Cells containing specific granules.—Single cells or small groups of cells containing granules, measuring up to 2 μ in size, were scattered throughout the epithelial outgrowths. The number of these granules varied: some cells contained only a few whilst others had about 10. Phase contrast microscopy revealed them to be distinctly outlined structures light gray in colour.

They gave a positive P.A.S. reaction and were resistant to diastase. Staining for lipids gave a negative result.

Time-lapse cinéphotography in phase contrast revealed irregular movements of the mitochondria and small granules in the cytoplasm, rotation of the nuclei [3, 7] and changes in the appearances of the nucleoli [2] in all three types of cell.

The present investigation revealed that ciliated and secretory cells occur in epithelial membranes grown from tissue cultures of epithelium of the rabbit Fallopian tube. Nothing was found to suggest that ciliated cells may be transformed into secretory cells and vice versa. Nor were cells showing both ciliary and secretory activity identified.

A more detailed paper will appear later.

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ULTRASTRUCTURE OF NUCLEAR MEMBRANE OF PLANT CELLS

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Electron-microscope studies of various types of animal cells have shown that the nuclear membrane is a double-layered fenestrated envelope with raised annuli surrounding "pore-like" areas [1–6, 8 10, 12, 15, 17]. Comparable structural patterns have not been described for nuclear membranes of plant cells. On the contrary, some microscopists have suggested from observations with the ordinary light microscope on fixed and living materials that nucleus and cytosome are separated merely by a phase boundary [7, 13, 16]. Since the observations on living cells were made using staminate-hairs of *Tradescantia*, it seemed desirable to utilize the modern

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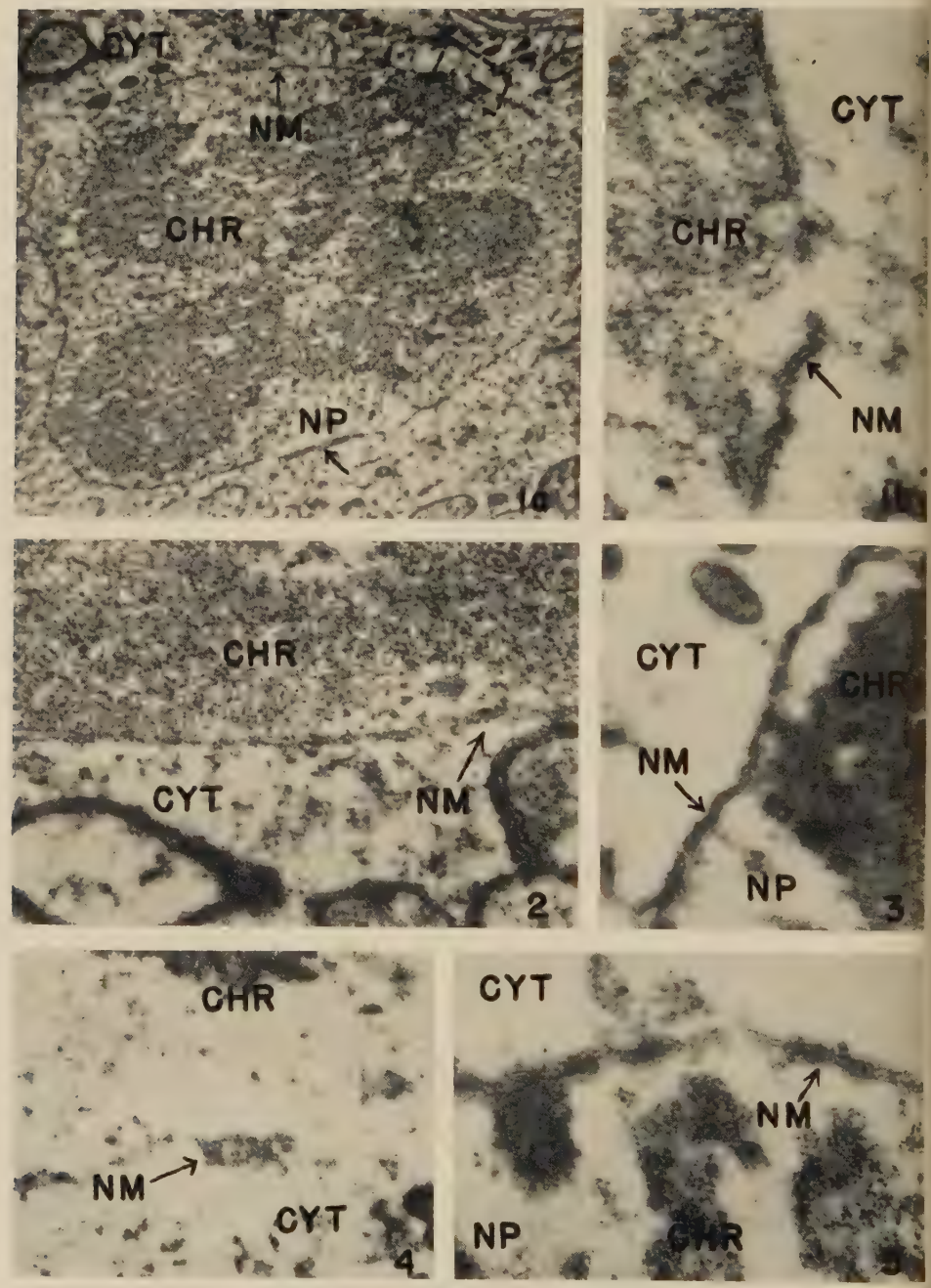
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chniques of electron microscopy for study of ultrathin sections of their cells. The dings are reported here, together with supplementary studies on the nuclear membrane in microsporocytes of *Tradescantia*.

Materials and methods.—Staminate-hair cells and pollen mother cells of *Tradescantia flexa* Raf. were used in this study. The hair cells are arranged end to end in single e, so that problems of fixation, dehydration and imbedding should be minimized compared with those encountered in preservation of cells aggregated into compact ssues. In the anther lobes a rapid exchange of liquids was assured by repeatedly ncturing the wall with a sharp glass needle.

Various fixatives containing osmium tetroxide in acetate, phosphate and veronal uffers at different pH's were used. Dalton's chrom-osmic fixative was also tested. some cases, the hair cells were frozen in the fixative. The fixed and dehydrated aterials were imbedded in a mixture of butyl and methyl methacrylate (9:1) and e blocks were sectioned with a Porter–Blum microtome. The sections were observed ithout removing the imbedding material.

Observations.—The cells of somatic and meiotic divisions were examined. A nuclear embrane was observed in prophases of staminate-hair cells (Fig. 5), and in diplotene ig. 1), diakinesis (Figs. 3–4) and first division telophase (Fig. 2) stages of the pollen other cells.

At diplotene and diakinesis (Figs. 1, 3, 4), the membrane appears interrupted, ossibly presaging its dissolution as the cell approaches metaphase. In first division lophase, no such interruption could be found (Fig. 2).

In cross-sectional view, the membrane appears as a double layer with two parallel ark lines separated by a non-electron scattering zone, which may be called a erinuclear space (Figs. 1*b*, 2, 3 and 5) according to Watson [17]. The perinuclear pace appears in places very narrow (100 Å) and elsewhere much broader (250 Å), ut normally its diameter is about 140 Å. In tangential view the membrane shows reticulate structural pattern (Fig. 4). Highly electron scattering materials form a ng around the less electron dense "pores" which are approximately circular, being lliptical in oblique section. The outside diameter of the pore is about 650 Å, the nside diameter being 380 Å, the distance from center to center of the pores is about 000 Å. The total thickness of the membrane in cross section is about 310 Å.

Electron micrographs of ultra-thin sections of cells of *Tradescantia reflexa*. Figs. 1-4, pollen nother cells. Fig. 5, staminate-hair cell.

Abbreviations: *CHR*, chromosomes; *CYT*, cytoplasm; *NM*, nuclear membrane; *NP*, nucleoplasm.

ig. 1. Diplotene stage, fixed in Dalton's chromo-osmic, pH 7.5. (*a*) Over-all view of the cell howing chromosomes and nucleoplasm surrounded by a double-layered membrane. The upper rrow indicates the membrane in cross section, the lower arrow shows the membrane in oblique ection. The black framed porton is enlarged in Fig. 1*b* to show the details. × ca. 7300; (*b*) Higher agnification of section framed in Fig. 1*a*. The arrow clearly shows the double-walled membrane. × ca. 50,000.

ig. 2. Telophase stage first meiotic division. Fixed in Dalton's chrom-osmic, pH 7.0. Arrow ndicates the granules of the membrane. × ca. 50,000.

ig. 3–4. Diakinesis stage. Fixed in phosphate-buffered OsO_4, pH 7.5.

ig. 3. Cross section of the nuclear membrane. × ca. 48,000.

ig. 4. Tangential view shows reticulate pattern of pores. × ca. 17,600.

ig. 5. Somatic prophase stage. Frozen in phosphate-buffered OsO_4, pH 7.5. Arrow indicates the nembrane. × ca. 50,000.

The thickness of the membrane and the dimensions of the pores are constant in both somatic and meiotic nuclei and the pores are equally spaced in both.

In some cases, the cross sectional view of the membrane shows small granules in both outer and inner layers (Fig. 2). These granules are about 90 Å in diameter and are distributed along the periphery of the pore.

Discussion. Antedating the electron microscope, vital stains, micro-piercing, phase, polarizing and ultra-violet microscopy were used to study the structure of the nuclear membrane. Callan and Tomlin [5] first described a detailed structural pattern of the nuclear membrane from studies with the electron microscope. Subsequently Bretschneider [4], Sjöstrand and Rhodin [15], Gall [8] and Hartmann [10] reported the same structural pattern as reported by Callan and Tomlin. In the following years several papers [1, 3, 6, 9, 12] appeared, clearly demonstrating the so-called "porosity" of the membrane. Watson [17] pictured the structure of the membrane in various types of rat cells. He reported that the structural organization of the membrane is the same in different types of tissues.

In a previous publication the nuclear membrane in cells of *Tradescantia* was described by Kaufmann and De [11] as a fenestrated double-walled structure. The present paper confirms and extends that report. In general, these observations are in accord with those of the nuclear membranes of animal cells. Earlier observations by Scott [14], using the light microscope, suggested the existence of perforations in the nuclear membranes of endosperm and nucellar tissues of the plants, *Acanthus* and *Tropeolum*, which may be similar to those now revealed by the electron microscope, but which were not clearly defined since they lay so close to the limits of resolution offered by the light microscope.

The observations given here on the nuclear membrane of the staminate-hair cell of *Tradescantia* stand in opposition to Wada's assumption about the absence of such membrane [16]. The presence of a well-defined morphological structure with details similar to those observed in animal cells, suggests that the nuclear membrane of both plants and animals is fundamentally the same, insofar as the structural pattern is concerned.

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QUANTITATIVE CHANGES IN SOLUBLE PROTEIN ANTIGENS DURING EARLY DEVELOPMENT OF THE SEA URCHIN[1]

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OLUBLE antigens in developing sea urchin embryos have previously been studied ualitatively [9, 7, 3]. The present study was undertaken in order to determine uantitative developmental changes in certain of the antigens extractable from the g and embryos. Since the technique of Oudin [5, 6] allows determination of relative tigen concentrations even in rather complex mixtures [10], it seemed well suited r this investigation. This paper represents a preliminary report of the results of e program which has been carried out in our laboratory since 1953.

Eggs of the sea urchin *Paracentrotus lividus* were collected at the Zoological Station Naples. Eggs from several females were pooled, fertilized and cultured. Embryos ere harvested at various stages of development, and aliquots of the samples were unted so that serological tests could be carried out on extracts from known numbers embryos. The material was frozen prior to extraction. The procedure for the quanti- tive extraction of soluble antigens which was finally adopted was the following: omogenization and repeated extractions in large amounts of buffered (pH 7.4) 15 *M* saline, centrifugation, dialysis against H_2O and concentration of the extract y lyophilization. The resulting dried material was redissolved in a small volume of uffered saline to give a final concentration of 150,000 eggs (embryos) per ml solu- on. The whole procedure was carried out in the cold. Duplicate extractions were ade on each of the samples. Nitrogen determinations were performed after ap- opriate steps in the procedure. Rabbit antisera against saline suspensions of omogenates from unfertilized eggs and developmental stages were prepared as reviously described [8].

For the serological determinations small bore glass tubes [6] were used. The wer portion was filled with 0.25 per cent saline agar containing antiserum in varying ncentrations. This was overlayed with the antigen solution to be tested. The tubes ere sealed and stored at 20°C. As the antigens diffused, bands of antigen-antibody recipitates formed and slowly migrated down the antiserum-agar column. The istance of the leading edge of the bands from the interphase boundary was measured t different times and plotted against the square root of the migration time. In his way a straight line was obtained for each precipitate. The slope of these lines hen gave a measure of the diffusion rates.

When extracts from unfertilized eggs and developmental stages were tested with ifferent antiserum samples, a number of bands appeared in the gel phase. Two of

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these were selected for study, because at constant antiserum concentration they were readily identifiable by their characteristic densities, sharp leading edges and conveniently differing diffusion rates. Mutual dilution tests [6, 10] also served to identify the corresponding antigens in different extracts. Heating experiments and precipitation with $(NH_4)_2SO_4$ at different degrees of saturation suggested that both antigens were proteins of different physico-chemical properties.

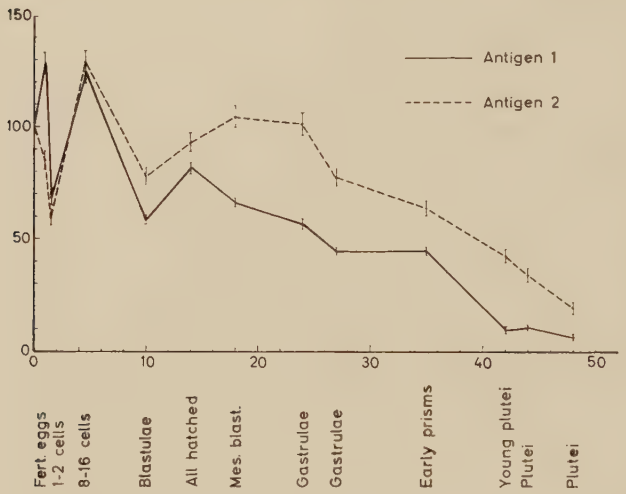

Fig. 1. Relative concentrations of antigens 1 and 2 in extracts from developmental stages of the sea urchin *Paracentrotus lividus*. Concentration in extract from unfertilized eggs = 100 p.c. Ordinate: percentage antigen. Abscissa: hours of development. For further explanation see text.

Serial dilutions of extract from unfertilized eggs were pipetted over duplicate series of antiserum-agar columns. When the resulting diffusion rates, determined as described above, were plotted against the logarithms of the antigen dilutions, straight lines were obtained for both antigens from 10–200 per cent antigen concentration (100 per cent: extract from 150,000 eggs per ml test solution). Highest accuracy was obtained by measuring the fast moving band (antigen 1) at 70 per cent and the slower moving band (antigen 2) at 30 per cent (v/v) antiserum concentration. Straight lines were also obtained with extracts from developmental stages. No statistically significant differences were found when the slopes of the diffusion rate/antigen dilution lines from different developmental stages were compared with the slopes from the unfertilized egg extracts. At appropriate antigen/antiserum ratios, the slopes of these lines have been shown to be a function of the diffusion coefficients of the antigens forming the corresponding precipitation bands [2]. Therefore, it could be concluded that no changes in the diffusion properties of our antigens took place during development.

On the basis of these findings the concentrations of the two antigens in extracts from different developmental stages were determined relative to the concentrations present in the unfertilized egg. The diffusion rates of the precipitation bands from a series of dilutions of unfertilized egg extract were plotted against the log of antigen dilution. By means of the standard lines so obtained (method of least squares), the antigen diffusion rates of extracts from equivalent numbers of embryos were then converted to relative antigen concentrations. The diagram shows the results of

typical experiment. The antigen content in an extract from unfertilized eggs 50,000/ml) was taken as 100 per cent and the antigen content in subsequent stages as expressed as the percentage of the unfertilized egg value. The points are average alues from two independent extractions of the same material. Each antigen was sted at its optimal antiserum concentration. In calculating the standard deviations ven in the diagram, account has been made for sampling and measuring errors and ıe error in the slope of the standard lines. Details of the calculations will be given ter. The results in this experiment were obtained with an anti-pluteus serum. ests with anti-egg serum gave identical results. Experiments with other develop- ental series showed a similar pattern of changes though the relative antigen levels uring cleavage and hatching varied somewhat from those illustrated in the diagram.

As can be seen from the diagram, in relation to the antigen content of the un- rtilized egg extracts, both antigens showed a marked decrease at the onset of cell ivision, followed by an increase up to the 16 cell stage. Moreover, antigen 1 had a op immediately after fertilization and antigen 2 a top at the mesenchyme blastula nd early gastrula stages. Following these changes in the cleavage stages, there was striking decrease in the amounts of both antigens during gastrulation, resulting in oncentrations of less than 20 per cent of the unfertilized egg in the plutei stages. nly to a slight degree did these changes reflect changes of general extractability f high molecular nitrogen as extracted by the procedure just described.

In order to ascertain the efficacy of the extraction method and to confirm the alidity of the results described above [1, 10], a quantitative adaptation of the bsorption technique was used [10]. Equal volumes of antiserum were absorbed with ifferent amounts of total unextracted homogenate and soluble and insoluble fractions om eggs and several developmental stages. All samples were homogenized, dialyzed nd lyophilized prior to absorption. The absorbed antisera were cleared by centri- ugation, mixed with saline-agar and tested in Oudin tubes with a concentrated tandard antigen solution. Since the diffusion rates of the bands of precipitate in- rease when the antibody concentration of a serum is decreased (6) and since different mounts of antibody had been precipitated by the various absorptions, the diffusion ates obtained with the absorbed antisera reflected the absorbing power and hence he antigen content of the preparations used for absorption. Plotting the diffusion ates against the amounts of absorbens used gave straight lines of different slopes, ll lines intersecting at the same point (0 absorption). From these slopes and the itrogen values of the preparations, the relative antigen content of homogenates nd fractions was calculated. In all samples tested the antigen content of the extracts vas approximately 95 per cent of that of the unextracted homogenates. Correspond- ngly, the insoluble fractions obtained by the extraction procedure mentioned above howed almost no antigen activity. Relative concentrations of the two antigens as letermined by the absorption method on total homogenates of different develop- nental stages confirmed the results of experiments with extracts as given in the diagram.

The question arises as to whether the developmental changes reported in this paper were due to actual changes in antigen quantities, to solubility changes or to both. If solubility changes were involved, the antigens upon transforming to the nsoluble state must have reacted in such a way that no free antigenic sites remained available for the serological reaction. However, it seems justified to explain the

developmental changes, at least in part, as reflections of specific metabolic activities of the embryo (cf. [4]). The antigens might thus be considered as yolk proteins which are utilized during differentiation. This concept is supported by cell fractionation experiments on unfertilized eggs. These experiments indicated, that both antigens were localized mainly in the yolk granules of the cell.

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TECHNICAL CONSIDERATIONS ON QUANTITATIVE MICRORADIOGRAPHY ON BONE

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During recent years the quantitative distribution of mineral salts in bone has been studied by various authors using the technique of quantitative microradiography introduced by Engström [2].

The general procedure has been that used by Amprino and Engström [1] and later by Owen [3], all of whom eliminated the necessity for determining the thickness of sections by making absorption measurements on closely adjacent structures only, and assuming a constant thickness. They then referred their readings to the absorption in a reference system which usually consisted of aluminium foils.

In our current bone studies, the necessity of investigating mineralisation over larger areas required an accurate determination of section thickness, which in bone specimens prepared for microradiography is usually of the order of 100 microns. This has been done with the aid of a profile microscope to an accuracy of ± 2 microns. The thickness of the specimen was measured by cutting the section with a microdissecting knife, and the thickness of the cut edge was then measured in the profile microscope, either on a single point or on equidistant points along the edge. These points could subsequently be localized fairly accurately in the microradiogram.

'Monochromatic" radiation was used in making the microradiograms, and the otographic density was kept within those limits in which the density response was ear with respect to incident X-ray energy. Hence the reference system could be itted.

Although the method outlined above does not provide optimum conditions for otometry, we prefer to use it since we have found it difficult to eliminate the evenness of intensity in a large field. We have also found that, due to "inhomoneities" in the foils, a reference system made of aluminium lacks the accuracy that inherent in the method itself.

A highly stabilized X-ray unit (Philips PW 1010) was used as radiation source d a copper target tube was operated at 20 kV. The radiation was filtered through 18 micron thick Ni-foil. X-ray spectroscopic analysis of the transmitted beam owed that more than 80 per cent of the radiation was within the range of 1.5-1.6 Å, us corresponding to the wavelength of a comparatively pure Cu $K\alpha$ emission line 54 Å). The straight line relationship between actual mass in the different steps of eference system and the absorption of X-rays in the same reference system further owed that the radiation was sufficiently monochromatic to obey the laws for sorption of strictly monochromatic X-rays. The distance between the target and e sample-film sandwich was 30 cm and provided a relatively even field of illumination. For the wavelength 1.54 Å, the photographic density response of the emulsions ed (Eastman Kodak Spectroscopic Plates GH 649 emulsion numbers 499 and 502) as found to be linear in relation to X-ray intensity up to a photometric density 0.7.

Microradiograms were developed for five minutes in undiluted D 19 B at 18°C d further processed in routine fashion. Densitometry was performed using a Zeiss Schnellphotometer" equipped with optics which minimized the influence of e Schwarzschild–Villiger effect. Photometric readings were made on a scale with 00 divisions, and scale deflections could be read within half a division.

In the absorption formula for monochromatic X-rays, $I = I_0 \cdot e^{-\mu \cdot t}$ (in which = transmitted intensity, I_0 = original intensity, e = base of natural logarithm, = linear absorption coefficient, t = thickness in cm), we now substitute the values r I and I_0 by densitometric values, so that $I = k \log_{10} (i_0/i_s)$ and $I_0 = k \log_{10} (i_0/i_d)$, here i_0, i_s, and i_d represent, respectively, transmitted light intensity in the nonposed emulsion area, in the sample area, and in the direct beam area, and k is a nstant. It is thus possible to solve the value for the absorption index $\mu \cdot t$ and, king the section thickness into account, to compute the linear absorption coefcient.

In an effort to analyze the various errors associated with this method, a "physical" odel experiment was designed. As a test object we used a sheet of fused quartz ne cm in length and 0.5 cm in breadth. One half of this sheet was 161 microns thick nd possessed a plane parallel tolerance of less than half a wavelength of visible ght. The other half of the sheet was wedgeshaped with an inclination of 161 to 0 microns in 5.0 mm.

The reproducibility of the densitometric method outlined above over an area with nstant thickness was computed from the μt values from 100 absorption measurements on the plane part of the sheet on one single microradiogram. The reproducibility ith slight changes in photographic density was computed by making 10 measure-

ments on each of ten different microradiograms varying in photographic density from 0.4–0.6. The error associated with the combination of densitometry and the determination of thickness on a given point was calculated as the square-root of the sum of the squared standard deviations of the two independent methods, both giving values having a normal distribution. The accuracy of the determination of thickness and subsequent absorption studies on equidistant points was calculated by applying this technique on the wedge-shaped part of the sample with ten observations on ten different plates, the thickness ranging from 150 50 microns. Finally the smallest detectable mass difference was estimated by registering the smallest change in thickness of the quartz wedge which gave a readable change in the value of i_s (half a scale unit). The values of the above studies were all shown to have a normal distribution. The densitometer slit was opened up to include an area of 900 microns^2 at a magnification of × 33.

Table I

Reproducibility

	Number observations	Linear absorption coefficient mean value	Standard deviation	Standard deviation per cent of mean
Densitometry on constant thickness 161 microns	100	80	±0.70	0.90
Densitometry on constant thickness, varying density	10 × 10	81	±1.30	1.42
Densitometry + local thickness determination, average thickness of 100 microns	100	81	±1.66	1.66
Densitometry + thickness determination on equidistant points, thickness varying from 50–150 microns	10 × 10	81	±2.60	3.25

Sensitivity

Number of observations	Average thickness in microns	Mean value in microns of smallest detectable difference in mass at optimal conditions	Standard deviation	Mean value in per cent of total mass	Transmission
50	150	1.28	0.45	0.85	0.30
50	100	1.06	0.32	1.06	0.44
50	50	0.85	0.23	1.70	0.62

The values recorded are listed in Table I. The systematic errors associated with the microradiographic method due to impure radiation were calculated from the mean value of ten mass determinations made on the plane part of the quartz sheet

n strictly monochromatic radiation and with the intensities recorded directly in eiger Müller tube fed to a rate meter and recorder. It was found that the values ained by the microradiographic method were somewhat too high, being of the er of + 5 per cent.

t is evident from the table that the microradiographic method for mass deter- ation used by the authors is capable of determining differences in mass of ± 1.1 cent at an average thickness of 100 microns. This figure is only slightly higher n the reproducibility of the densitometry procedure which is ± 0.9 per cent. The hod of thickness determination used by the authors to get absolute values for eralization of bone at an average thickness of 100 microns somewhat decreased accuracy of mass difference determination. Thus depending upon whether the kness was measured on easily defined structures or along equidistant points, the allest detectable difference in mass was calculated to be ± 1.66 or ± 3.25 per cent, pectively.

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THE EFFECT OF THYROXINE ON THE SWELLING OF MITOCHONDRIA ISOLATED FROM NORMAL AND NEOPLASTIC LIVERS

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contrast to the mitochondria isolated from rat diaphragm, heart, spleen, brain d testis [7], rat liver mitochondria have been reported to undergo a profound elling on addition of thyroxine [5]. It has now been found that mitochondria iso- ed from primary azo dye-induced liver tumors of mice and rats have largely or mpletely lost the ability to swell in the presence of thyroxine.

Female CBA mice were fed *o*-aminoazotoluene (*o*-AAT) and female rats of the bred strain R Amsterdam received *p*-dimethylaminoazobenzene (*p*-DAB) together th a diet deficient in proteins and vitamin B. Mitochondria were isolated (10 min. 5000 *g*) and washed twice in 0.30 *M* sucrose containing 0.001 *M* ethylenediamino- traacetate (EDTA) of pH 7.4. Suspensions were made in 0.30 *M* sucrose buffered ith 0.02 *M* Tris (pH 7.4) and incubated at room temperature in the absence and esence of DL-thyroxine (3×10^{-5} *M*). The spontaneous and thyroxine-induced vellings of the mitochondrial samples, including those of normal animals under arious experimental conditions, are listed in Tables I and II as the percentage de- ease in the optical densities at 520 mμ. In a few experiments the mitochondria ere isolated in the absence of EDTA.

TABLE I

Spontaneous and thyroxine-induced swelling of mitochondria isolated from normal and neoplastic rat livers.

Mitochondria isolated and incubated as indicated in the text. The optical densities of the individual suspensions varied from 0.480–0.540 at 520 mμ.

Δ D520 controls = percentage decrease in the optical densities of the control suspensions incubated over a period of 45 minutes. Δ D520 thyroxine = percentage decrease in the optical densities of the suspensions containing DL-thyroxine (3×10^{-5} *M*) less that observed for the controls over the same period. Values within parentheses are those found for mitochondria which had been isolated in the absence of EDTA.

Tumors were dissected free from adhering liver tissue. Classification: solid (hepatocellular) and adenomatous (cholangiocellular) hepatomas. These terms have been used only in a descriptive sense.

Mitochondria from	Δ D520 controls	Δ D520 thyroxine
Liver, normal rat (strain R Amsterdam)	0 (12)	44 (30)
Liver, rat—protein and Vit. B-deficient diet fed 5 months	0 (24)	44 (10)
Liver, rat—same, 9 months	4 (4)	40 (35)
Liver, rat fed *p*-DAB 5 months	0	30
BY266—massive tumoroid hyperplasia with few hepatocellular carc. cells	5	12
BY240—solid hepatoma	5	0
BY279—solid hepatoma	8	2
BY265—solid hepatoma	1	8
BY275—mixed adenomatous–solid hepatoma	0	0
BY276—adenometous hepatoma	5	0
BY231—adenomatous hepatoma	6	14
BY227—adenomatous hepatoma, first transplant	5	0
Liver of BY 266	14	14
240	13	17
279 (various degrees of fatty infiltration, cirrhosis, and	8	12
265 cellular and nuclear polymorphy)	4	20
275	6	16
276	3	20

The data clearly illustrate that thyroxine has a greater effect on normal liver mitochondria than on mitochondria isolated from mouse livers in the early stage of carcinogenesis or from mouse and rat livers in which tumors were present.

The swellings of the various rat liver mitochondria were always strongly counteracted by 2,4-dinitrophenol (10^{-4} *M*) thus confirming the results of Tapley [5] concerning normal rat liver particles. The swelling of the mouse liver mitochondria under the various experimental conditions were not always counteracted by 2,4-DNP as indicated in Table II. This was especially significant for aged mitochondria.

The possibility that the lack of thyroxine to produce swelling of the *tumor* mitochondria might have been due to the fact that the particles were already markedly swollen before the addition of the hormone, could be ruled out on account of observations made with the phase contrast microscope.

TABLE II

ntaneous and thyroxine-induced swelling of mitochondria from normal and neoplastic mouse livers.

Mitochondria from	Δ D520 controls	Δ D520 thyroxine
er, normal mouse (CBA)	2 (13)	38 (30)
er, mouse fed *o*-AAT 7 months (2 animals) } microscopic neo-	2 (18)	20 (12)
er, mouse fed *o*-AAT 7 months (2 animals) } plastic foci	2	20
er, mouse fed *o*-AAT 11 months (3 animals) : multicentric neo-		
plastic foci	(18)*	(12)*
patomas, from 5 animals[a]	18*	3
patomas, from 3 animals[a]	6	9
A 224—hepatoma, first transplant	0	4
A 71—hepatoma, ninth transplant	2	9
er, hepatomas removed[b] } multicentric neoplastic foci	2	16
er, hepatomas removed[b] }	2	17
er, normal mouse (after aging 60 min. at 37°C in 0.30 *M* sucrose)	(26)*	(10)*
er, normal mouse (after aging 60 min. at 37°C in 0.30 *M* sucrose–0.001 *M* EDTA)	26*	14*

* Not counteracted by 2,4-DNP (10^{-4} *M*).
[a] Corresponding animals.
[b] Corresponding animals.

Further experiments demonstrated that sucrose or sucrose-EDTA prepared itochondria from many other tumor strains were also completely refractory to the tion of thyroxine (measurements of optical densities and phase contrast micro-opical observations). We have shown earlier [1–3] that mitochondria from certain mor strains, including the rat hepatomas (designated, for convenience, as type I itochondria) are biochemically less intact than other tumor mitochondria (type II). the present experiments thyroxine induced a swelling in some of the type II itochondria, e.g. from the mouse hepatomas T28012 and T26473 (transplanted om spontaneous tumors), the ovarian tumor T5441 and the testicular tumor T26554, nly. The latter swellings were however much smaller than those obtained with ormal liver mitochondria. In this connection (compare [5, 6]) it may be of interest at thyroxine has also been found [4] to uncouple the oxidative phosphorylations certain type II-tumor mitochondria (e.g. from T26473 and T5441, but not from 28012) under conditions in which the hormone did not affect the corresponding rocess of normal rat and mouse liver mitochondria.

It was observed in the course of this investigation that on addition of type I-tumor itochondria to normal mouse liver mitochondria, the thyroxine-induced swelling f the latter was completely or almost completely inhibited. Type II mitochondria ere very less active in this respect.

Apart from the loss of response to thyroxine, the combined liver-tumor (type I) itochondrial system is also unable to oxidize fatty acids [1–3]. The adenosine tri-

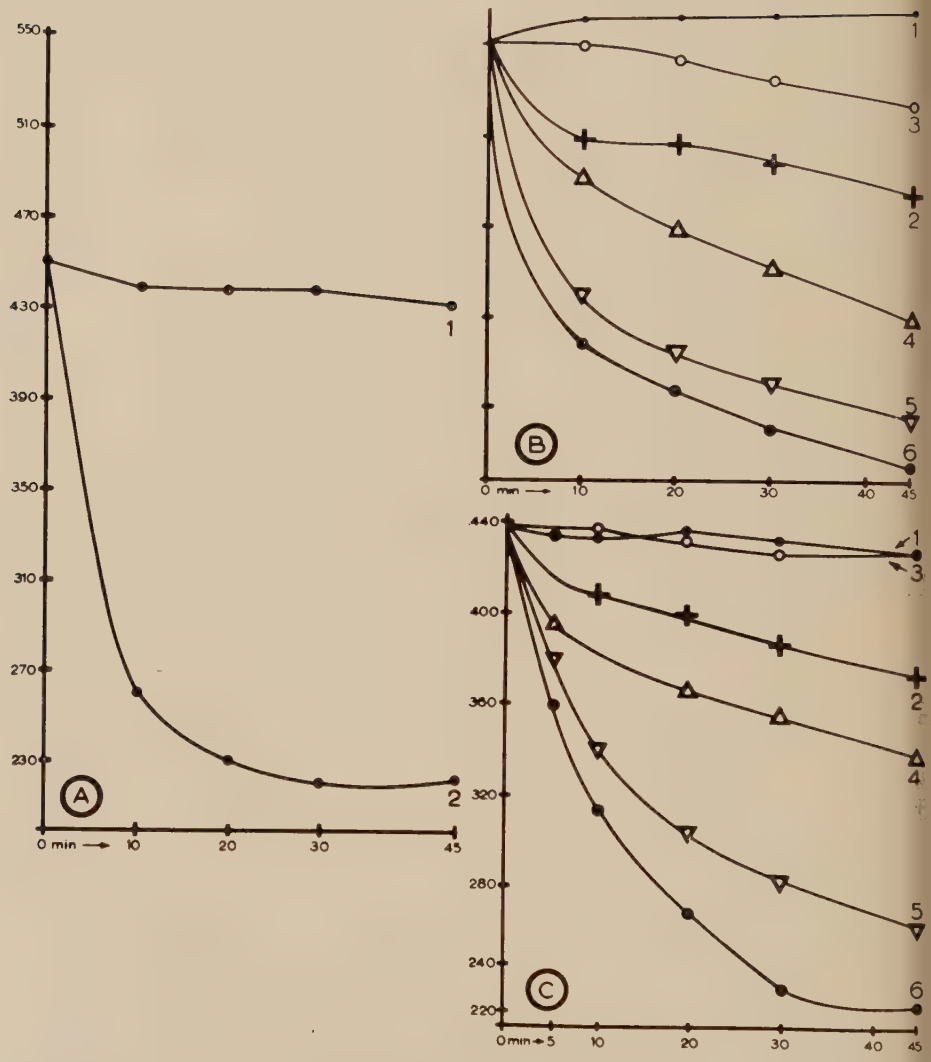

Fig. 1. Effect of thyroxine on the swelling of mouse liver mitochondria under various conditions. (A) Freshly prepared liver mitochondria. (B) Freshly prepared liver mitochondria in the presence of a small amount of mitochondria isolated from the adrenal cortex carcinoma T17572. In the present experiment thyroxine had still some effect on the optical densities (curve 2); in other experiments this was completely absent. (C) Liver mitochondria (from the same pool as those from experiment A) after they had been kept for 2 hours in the sucrose-EDTA suspension at 0°C.

Curves: **1**, control; **2**, DL-thyroxine (3×10^{-5} *M*); **3**, thyroxine + 2,4-dinitrophenol (10^{-4} *M*); **4**, thyroxine + NAA (5×10^{-3} *M*); **5**, thyroxine + DPN (10^{-3} *M*); **6**, thyroxine + ATP (2×10^{-3} *M*). (Curve 2 in Fig. 1 B and C represents approximately the course of the optical densities of the suspensions incubated in the presence of thyroxine + ATP + 2,4-DNP.)

sphate (ATP)- and diphosphopyridine nucleotide (DPN)-splitting activities of tumor mitochondria were responsible for this lack of oxidation, which could be tly repaired by inhibiting the latter enzymes. Therefore it was tried in the present eriment to reestablish the lost thyroxine effect in the combined liver-tumor ochondrial suspensions by adding the DPNase inhibitor nicotinamide (NAA), N or adenine nucleotides. As shown in Fig. 1A and B, NAA, DPN and ATP were ctive in restoring the thyroxine effect in increasing order. Adenosine monophosite was less and adenosine diphosphate was somewhat more active than ATP (obied from Zellstoff-fabrik, Waldhof, Germany). In the absence of thyroxine none he compounds produced any swelling of the mitochondria. However, doubt may raised as to the specificity of these effects since ATP from a purer quality (Sigma) not give rise to such a pronounced effect as that illustrated in Fig. 1B, curve 6. e possibility is thus at hand that some impurity in ATP, and perhaps also in DPN, entiated the thyroxine effect. When in addition to ATP, 2,4-DNP was also present the combined liver-tumor mitochondrial system the effect of thyroxine was aboied again. On separate incubation of type I-tumor mitochondria in the presence of AA, DPN or ATP, thyroxine had no effect at all on the optical density of the spensions. This indicated that in the NAA, DPN or ATP-fortified liver-tumor tochondrial suspensions the swelling of the liver particles had been restored.

Mouse liver mitochondrial suspensions kept for 2–3 hours at 0°C exhibited optical nsities which did not differ from those of the fresh suspension indicating that no ontaneous swelling had taken place. Yet the addition of thyroxine to the aged spensions resulted only in a small drop of the optical density. Since these suspenns are still capable of oxidizing fatty acids, any lesion in their biochemical constition must be minute. As described above NAA, DPN and ATP restored the yroxine effect (Fig. 1C). 2,4-DNP again counteracted the effect of thyroxine under e latter conditions.

In conclusion it may be stated that, whatever the precise mechanism of action ay be, the observed effects of NAA and the nucleotides in conjunction with thyxine reveal differences among the organizations of "damaged" liver mitochondria d type I-tumor mitochondria (including those from rat hepatomas). Whether this is its origin in the fact that the latter are more severely damaged from the biemical point of view than the former or in the fact that the thyroxine-sensitive ctor is naturally absent from these tumor mitochondria cannot be decided upon the basis of this investigation. Although generalizations are not likely to be arranted it may nevertheless appear from the present experiments that in order be active, thyroxine has to interact with some component of the *integrated* biemical structure of the liver mitochondria.

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THE NUCLEUS AND RIBONUCLEIC ACID SYNTHESIS IN AMOEBA[1]

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In a currently considered scheme of events, ribonucleic acid has been proposed as the carrier of genetic specificity from the desoxyribonucleic acid of the chromosomal gene of the nucleus to sites of protein synthesis within the cytoplasm. As a part of this thesis it is necessary to demonstrate that the nucleus is (*a*) capable of synthesis of RNA, and (*b*) that such nucleus-synthesized RNA is transferred to the cytoplasm. James [3] has given strong evidence that the nucleus is necessary to maintain the RNA level in the cytoplasm of amoebae. The clear-cut experiments of Goldstein and Plaut [2] involving the transplantation of isotopically labelled nuclei in amoebae present the most conclusive evidence yet available that the nucleus does synthesize RNA and releases it to the cytoplasm. The possibility remains, nevertheless, that the cytoplasm is also capable of RNA synthesis, and in fact, Brachet and Szafraz [1] have reported the incorporation of orotic acid into RNA of enucleate fragments of the unicellular alga, *Acetabularia mediterranea*.

The experiments reported here are concerned with the comparative ability of nucleated and enucleated amoebae to incorporate C^{14}-uracil into RNA. *Amoeba proteus* was cultured on *Tetrahymena* as described in [7]. Several hundred amoebae were removed from a culture, washed, and then starved for 24 hours at 18°C to rid them of the major portion of food vacuoles. Two hundred amoebae were cut into nucleated and enucleated halves. Whole cells served as an additional control. Immediately following the cutting, whole cells and nucleated and enucleated halves were placed in inorganic medium [5] to which C^{14}-uracil had been added (0.2 μc/ml final concentration) and remained there for 12 to 120 hours. At intervals of 12, 24, 48, 72, 96, and 120 hours groups of nucleated and enucleated halves and whole amoebae were removed from the uracil solution and washed free of all external radioactivity. Fifteen amoebae from each group were placed on planchets for assay of total radioactivity taken up. The remaining amoebae were then placed on duplicate sets of clean, albuminized microscope slides, squashed with a coverslip, and fixed (1:1 mixture of 45 per cent acetic acid and 95 per cent ethyl alcohol). These were then washed in distilled water for two hours with frequent changes of water in order to wash out unincorporated uracil. The slides were covered with Kodak stripping film. Of each of the duplicate sets of slides one was allowed to expose for 3 weeks and the other for 6 weeks. The autoradiographs were developed in Kodak D-11 developer for 10 minutes at 25°C and examined with phase contrast microscopy.

The amoebae placed on planchets were assayed with a windowless Geiger counter for total amount of C^{14}-uracil taken up. These data are presented in Fig. 1 and show the accumulation of radioactivity by all three groups of amoebae over a 120-hour period. The results agree with the findings of Plaut and Rustad [6] on the uptake of C^{14}-adenine by amoebae over a 51-hour period to the extent that the activity in nucleated halves is 70 per cent to 80 per cent of the amount accumulated by whole cells

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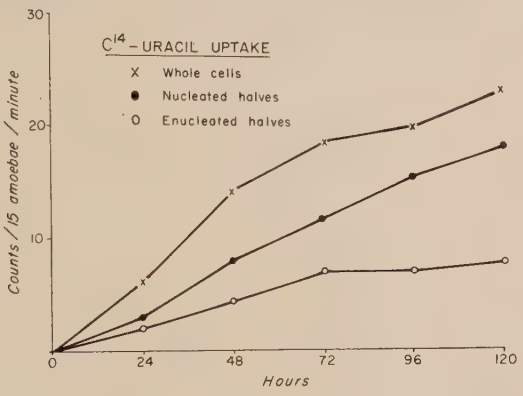
ile enucleated fragments take up only 35 per cent of the whole cell amount. The nparison of radioactivity levels in the various groups indicates that the amount uptake is not strictly proportional to cytoplasmic volume but is influenced by the sence of the nucleus. Furthermore, the uptake in whole amoebae and nucleated lves is continuous while the enucleated groups show a levelling-off after 72 hours. is levelling-off in the enucleate curve suggests the attainment of an equilibrium of



g. 1. Uptake of C^{14}-uracil by enucleated d nucleated halves and whole amoebae er a 120-hour period.

nbound C^{14}-uracil between the enucleate cytoplasm and the outside medium. Cells ith nuclei continue to accumulate radioactivity apparently because uracil is con- antly withdrawn into RNA, thus permitting further inward diffusion of uracil. his interpretation is supported by the autoradiographic results (see below).

An essential point is the demonstration of C^{14}-uracil uptake by enucleated amoebae; he failure of incorporation of uracil into RNA (see below) cannot be ascribed to an npairment in the uptake mechanism. Whole amoebae and nucleated halves show eak nuclear autoradiographs with 12 hours of incubation in the uracil solution, but o detectable activity has appeared in the cytoplasm. With 24 hours of incubation he nuclear autoradiograph is strong, and by 48 hours the cytoplasm is weakly but early radioactive. At the end of 120 hours, whole and nucleated halves of amoebae now strong nuclear and cytoplasmic autoradiographs. More than 95 per cent of the ctivity can be removed by treatment with ribonuclease. Enucleated amoebae, owever, even after 120 hours in the active solution, show absolutely no incorporation f radioactivity. The extremely low background of silver grains on the stripping lm would permit detection of even a very low level autoradiograph over the enu- leated fragment.

Although enucleated amoebae are clearly capable of uracil uptake, they are *ompletely incapable* of incorporating this precursor in RNA. This result, together ith the initial appearance of radioactivity in the nucleus and only later in the cyto- lasm, are taken as evidence that *all* the RNA found within the amoeba cytoplasm nd nucleus is elaborated within the nucleus. Perhaps the various reported effects f enucleation [4, 5] can eventually be traced to the loss of RNA synthesizing ability y such cells. In any case, these findings add support to the proposal of RNA as a nolecular communication from nucleus to cytoplasm. Extensions and further dis- ussion of these experiments will be reported in a subsequent paper.

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A SIMPLE MIDGET X-RAY TUBE FOR HIGH RESOLUTION MICRORADIOGRAPHY

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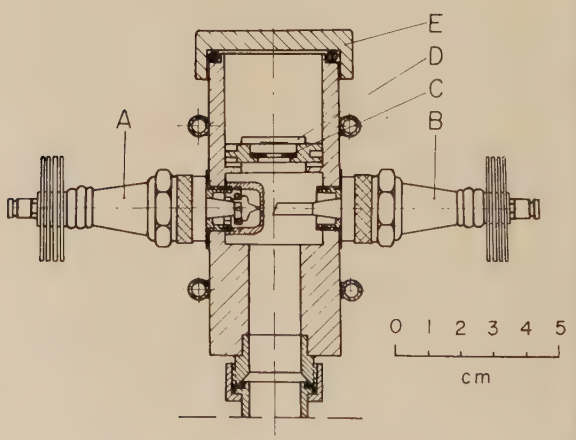
Recently it was shown [2] that the utilization of extremely soft X-rays permits the recording of X-ray images of cellular structures only a few tenths of a micron in size. For such purposes the X-rays have energies between 500 and 1500 volts. The technique of mounting the biological specimen directly on the collodion coated fine grained photographic emulsion reduces the geometrical unsharpness to such an extent, that the X-ray resolution of the microradiographic image is the same or better than



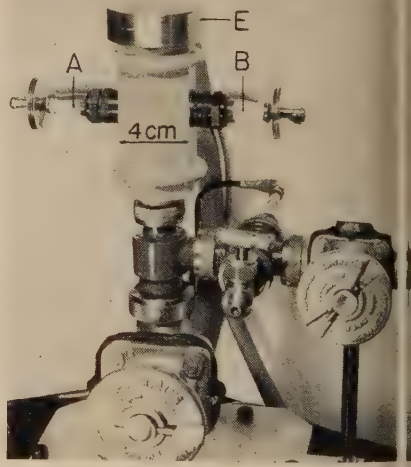
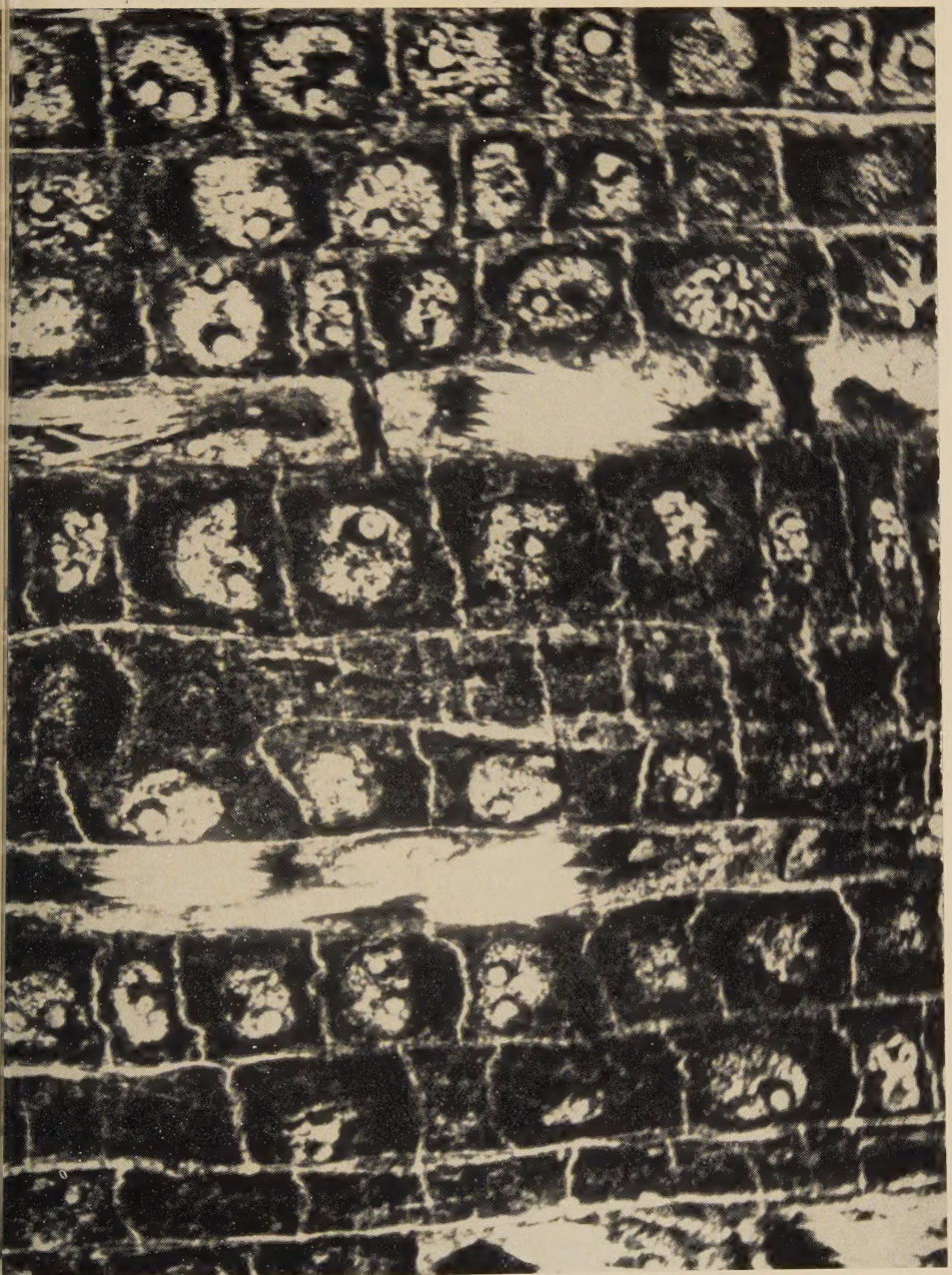


Fig. 1 (*left*). Cross section of the midget X-ray tube.
Fig. 2 (*right*). The tube mounted on a valve system on top of the high vacuum diffusion pump. *A*, cathode; *B*, target; *E*, removable lid. Cf. Fig. 2.



ig. 3. Microradiogram showing the distribution of dry weight within the nuclear structures in ells from *Allium cepa*. White areas absorb the X-rays. Note the highly absorbing calcium oxalate rystals. Mag. × 1180.

the maximum resolving power of the light microscope used to inspect the microradiogram [1, 3].

A simple, small X-ray tube which is continuously evacuated, has been constructed to record such high resolution microradiograms of biological specimens. The construction of this miniature tube is apparent from Fig. 1. The electrons from the filament in *A* are focused to a fine point on the target, *B*. The focal spot is approximately 0.1 mm in diameter. Two ordinary automobile spark plugs serve as insulated bases for the cathode and the anode, and are kept vacuum tight by lead gaskets. An approximately 1000 Å thick aluminum foil, *C*, protects the photographic emulsion from the filament light. A system of communicating channels prevents the breaking of this foil during the evacuation and opening of the X-ray tube. The sample film is mounted at *D*, when the cap *E* is removed. With this tube, also shown in Fig. 2, an X-ray area of about 12 mm in diameter is easily obtained. The exposure times are relatively short and the current through the tube is no more than about 1 mA. A result of microradiography utilizing this tube is shown in Fig. 3, where the distribution of dry weight within the nuclear structures during the various phases of the mitosis is clearly demonstrated in a section from the root tip of *Allium cepa*. The image was exposed at 1200 volts and 1 mA for 20 minutes. The tube has a by pass system of vacuum valves, Fig. 2. After introducing the specimen and doing the top *E*, the tube is evacuated and ready for operation within 30 seconds. Our experience indicates that the combined X-ray tube and microradiographic camera described in this note will become very handy in routine microradiographic studies of the distribution of the dry weight and water [1] in biological specimens.

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METHYLTHIOADENOSINE, A GROWTH FACTOR FOR *ENDAMOEBA HISTOLYTICA*

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A number of growth factors for *E. histolytica* have been detected by Nakamura and Baker [3]. Since numerous substances involved in nucleic acid metabolism were found to be ameba-stimulatory, pyrimidine amides were synthesized and tested against the amebas; most of the pyrimidine analogs were found to be inhibitory [1]. This report describes the ameba-growth-promoting activity of methylthioadenosine, which was obtained through the courtesy of Dr. F. Schlenk, Division of Biological and Medical Research, Argonne National Laboratories, Lemont, Illinois, U.S.A.

The assay method for evaluating the activity of methylthioadenosine was es-

tially identical with the methods reported earlier by us [3, 2]. The activity of compound was observed in an environment containing no living bacteria, there-e, the effects observed were those directly on *E. histolytica*. The stimulatory ac-n was recorded in terms of amebic multiplication; samples of ameba cultures re counted at the end of the incubation period. After the counts were made the ebas were subcultured into culture tubes containing similar nutritional ingre-nts and into culture tubes which were inoculated with the associated bacterial ra. All experimental tubes were inoculated with stock cultures of *E. histolytica* owing in association with a mixed bacterial flora; penicillin and streptomycin in ge quantities were included in all tubes to sterilize the bacteria introduced with e amebic inocula. Controls consisted of tubes containing the basal medium but king the compounds being assayed. A growth control consisted of tubes contain-g the basal medium and adenosinetriphosphate plus ribose-5-phosphate since ese two growth factors will promote growth of *E. histolytica* in the absence of cteria [2].

The ameba counts in tubes containing only the basal medium ranged from 0 to amebas per low power field (these tests were done in quadriplicate and two samples ere counted from each tube; the average counts and the range of counts were re-rded). The average count was 0.9 for the control. Those tubes containing the asal medium, adenosinetriphosphate, and ribose-5-phosphate had ameba counts nging from 125 to 169 with an average count of 153. Culture tubes containing the asal medium and methylthioadenosine (1.0 mg per ml) produced ameba cultures ith counts ranging from 195 to 230 with an average of 219 amebas per low power eld. Further addition of adenosinetriphosphate and ribose-5-phosphate to this ethylthioadenosine-fortified medium did not significantly increase amebic multi-lication. It is interesting to note that when adenosine (1.0 mg per ml) was added the basal medium, the ameba count was approximately 50 per low power field. herefore, it seems unlikely that the growth factor activity of methylthioadenosine due to the adenosine released upon hydrolysis and that it has some independent ctivity in the growth of *E. histolytica*. At this stage our knowledge of the metabo-sm of the amebas is insufficient to determine the role of this factor; further studies re being performed with related compounds in order to establish the site of ac-ivity of methylthioadenosine.

Summary.—Methylthioadenosine was found to be *in vitro*, under bacteria-free onditions, a potent growth factor for *Endamoeba histolytica*.

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ERRATUM

Runnström, J., *This Journal* **11**, 660–664 (1956).

Page 661, line 4 from bottom—*For* (8.5 ml isot. LiCl+91.5 ml sea water) *read* (3.75 ml isot. LiCl+96.25 ml sea water).

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